



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

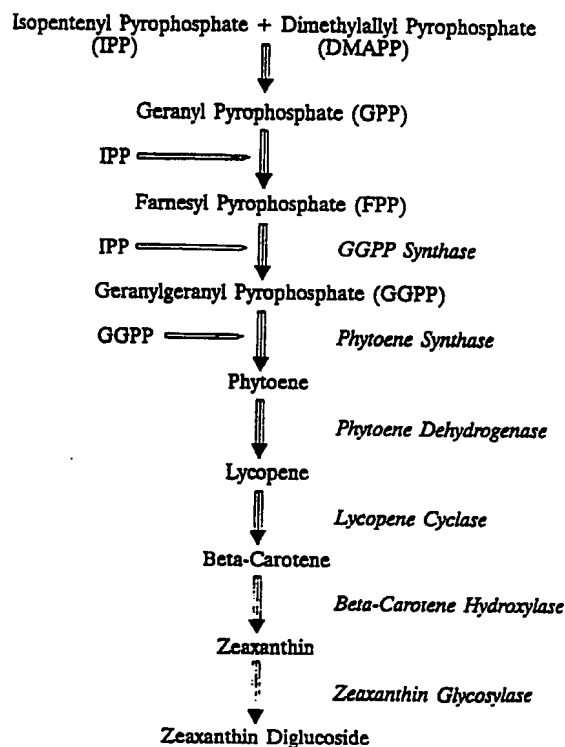
<p>(51) International Patent Classification ⁵ : C07H 15/12, C12P 21/00, 23/00 C12P 5/00, C12N 15/52, 15/70 C12N 15/74, 15/81</p>	<p>A1</p>	<p>(11) International Publication Number: WO 91/13078 (43) International Publication Date: 5 September 1991 (05.09.91)</p>
<p>(21) International Application Number: PCT/US91/01458 (22) International Filing Date: 4 March 1991 (04.03.91) (30) Priority data: 487,613 2 March 1990 (02.03.90) US 525,551 18 May 1990 (18.05.90) US 562,674 3 August 1990 (03.08.90) US Not furnished 28 February 1991 (28.02.91) US (71) Applicant: AMOCO CORPORATION [US/US]; Mail Code 1907, Patents and Licensing Department, P.O. Box 87703, Chicago, IL 60680-0703 (US). (72) Inventors: AUSICH, Rodney, Lee ; 158 Forest Street, Glen Ellyn, IL 60137 (US). BRINKHAUS, Friedhelm, Luetke ; 5885 Forest View Road #611, Lisle, IL 60532 (US). MUKHARJI, Indrani ; 2300 Central Park Avenue, Evanston, IL 60201 (US). PROFFITT, John, Houston ; 1008 N. Hayes Avenue, Oak Park, IL 60302 (US). YARGER, James, Gregory ; 221 Chasse Circle, St. Charles, IL 60174 (US). YEN, Huei-Che, Bill ; 1311 Jane Avenue, Naperville, IL 60540 (US).</p>		<p>(74) Agents: MICKELSON, Carole, A. et al.; Amoco Corporation, Patents and Licensing Department, Mail Code 1907, P.O. Box 87703, Chicago, IL 60680-0703 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>

(54) Title: BIOSYNTHESIS OF CAROTENOIDS IN GENETICALLY ENGINEERED HOSTS

(57) Abstract

DNA segments encoding the *Erwinia herbicola* enzymes geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and zeaxanthin glycosylase and DNA variants thereof encoding an enzyme having substantially the same biological activity, vectors containing those DNA segments, host cells containing the vectors and methods for producing those enzymes, a method for protecting plants from the herbicide norflurazon, as well as methods for producing GGPP and the carotenoids phytoene, lycopene, β -carotene, zeaxanthin and zeaxanthin diglucoside by recombinant DNA technology in transformed host organisms are disclosed.

Carotenoid Biosynthesis Scheme



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

-1-

**BIOSYNTHESIS OF CAROTENOIDS
IN GENETICALLY ENGINEERED HOSTS**

Description

5 Technical Field

The present invention relates to carotenoid biosynthesis. More specifically, this invention relates to the isolation, characterization and expression of the six Erwinia herbicola genes encoding the enzymes geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase that catalyze the formation of geranylgeranyl pyrophosphate and the carotenoids phytoene, lycopene, β -carotene, zeaxanthin and zeaxanthin diglucoside, respectively, each formed product (through zeaxanthin) being an immediate precursor for the next-named product. The invention also relates to methods for expression of these Erwinia herbicola enzyme genes in prokaryote hosts such as Escherichia coli and Agrobacterium tumefaciens, in eukaryote hosts such as yeasts like Saccharomyces cerevisiae and higher plants such as alfalfa and tobacco, as well as to methods for preparation of GGPP and those carotenoids.

Background Art

Carotenoids are 40-carbon (C_{40}) terpenoids consisting generally of eight isoprene (C_5) units joined together. Linking of the units is reversed at the center of the molecule. Trivial names and abbreviations will be used throughout this disclosure, with IUPAC-recommended semisystematic names given in parentheses after first mention of each name.

Carotenoids are pigments with a variety of applications.

Phytoene (7,8,11,12,7',8',11',12'- ψ octahydro- ψ , ψ -carotene) is the first carotenoid in the carotenoid biosynthesis pathway and is produced by the dimerization of a 20-carbon atom precursor, geranylgeranyl pyrophosphate (GGPP). Phytoene has useful applications in treating skin disorders (U.S. Patent No. 4,642,318) and is itself a precursor for colored carotenoids. Aside from certain mutant organisms, such as Phycomyces blakesleeanus carB, no current methods are available for producing phytoene via any biological process.

In some organisms, the red carotenoid lycopene (ψ , ψ -carotene) is the next carotenoid produced of the phytoene in the pathway. For example, lycopene imparts the characteristic red color to ripe tomatoes.

Lycopene has utility as a food colorant. It is also an intermediate in the biosynthesis of other carotenoids in some bacteria, fungi and green plants.

Lycopene is prepared biosynthetically from phytoene through four sequential dehydrogenation reactions by the removal of eight atoms of hydrogen. The enzymes that remove hydrogen from phytoene are phytoene dehydrogenases. One or more phytoene dehydrogenases can be used to convert phytoene to lycopene and dehydrogenated derivatives of phytoene intermediate to lycopene are also known. For example, some strains of Rhodobacter sphaeroides contain a phytoene dehydrogenase that removes six atoms of hydrogen from phytoene to produce neurosporene.

Of interest herein is a single dehydrogenase that converts phytoene into lycopene. That enzyme removes four moles of hydrogen from each mole of phytoene, and is therefore referred to hereinafter as

-3-

phytoene dehydrogenase-4H. The Rhodobacter phytoene dehydrogenase that removes three moles of hydrogen from each mole of phytoene will be hereinafter referred to as phytoene dehydrogenase-3H so that the distinctions
5 between the two enzymes discussed herein can be readily maintained.

Lycopene is an intermediate in the biosynthesis of carotenoids in some bacteria, fungi, and all green plants. Carotenoid-specific genes that
10 can be used for synthesis of lycopene from the ubiquitous precursor farnesyl pyrophosphate include those for the enzymes GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H.

Beta-carotene is the third carotenoid produced
15 in the Erwinia herbicola carotenoid biosynthesis pathway. It is also synthesized by a number of bacteria, fungi, and most green plants.

Beta-carotene has utility as a colorant for margarine and butter, as a source for vitamin A
20 production, and has recently been implicated as having preventative effects against certain kinds of cancers.

For example, prospective and retrospective epidemiologic studies have consistently shown that low levels of serum or plasma beta-carotene are associated
25 with the subsequent development of lung cancer. Because retinol is not similarly related to lung cancer risk, beta-carotene appears to have a protective effect without its conversion to vitamin A. Ziegler, Amer. Instit. Nutr., publication 022/3166/89, 116 (1989).

Beta-carotene is produced by the cyclization of unsaturated carotenoids in a procedure not yet well understood. Bramley et al, In Current Topics in Cellular Regulation 29:291,297 (1988). Because only
30 mutants that accumulate lycopene but not gamma-carotene
35 (another potential precursor) have been found, it is

believed that in both plants and microorganisms a single cyclase is responsible for conversion of lycopene to beta-carotene. Generally, the enzymes involved in this cyclization have been found as
5 integral membrane proteins.

Current methods for commercial production of beta-carotene include isolation from carrots, chemical synthesis [Isler et al., United States Patent 2,917,539 (1959)] and microbial production by hoanephora trispora
10 [Zajic, United States Patents 2,959,521 (1960) and 3,128,236 (1964)].

Zeaxanthin (β,β -carotene-3,3'-diol) is the fourth carotenoid produced in the Erwinia herbicola carotenoid biosynthesis pathway. Zeaxanthin, a yellow
15 pigment, is currently used as a colorant in the poultry industry.

Chemical synthesis methods for zeaxanthin production exist, but these are inefficient and not commercially competitive with the existing biomass
20 sources. Presently, the commercial sources for zeaxanthin are corn grain, corn gluten meal and marigold petals. The level of zeaxanthin in corn kernels averages about 0.001 percent (dry weight) and the level in corn gluten meal averages about 0.01
25 percent (dry weight). All these sources are characterized by low and inconsistent production levels.

Zeaxanthin diglucoside, the fifth carotenoid produced in the Erwinia herbicola carotenoid
30 biosynthesis pathway, is also useful as a food colorant and has a yellow color similar to that of zeaxanthin.

Carotenoids are synthesized in a variety of bacteria, fungi, algae, and higher plants. At the present time only a few plants are widely used for
35 commercial carotenoid production. However, the

-5-

productivity of carotenoid synthesis in these plants is relatively low and the resulting carotenoids are expensively produced.

One way to increase the productive capacity of biosynthesis would be to apply recombinant DNA technology. Thus, it would be desirable to produce carotenoids generally and zeaxanthin and its diglucoside specifically by recombinant DNA technology. This permits control over quality, quantity and selection of the most suitable and efficient producer organisms. The latter is especially important for commercial production economics and therefore availability to consumers. For example, yeast, such as S. cerevisiae in large fermentors and higher plants, such as alfalfa or tobacco, can be mobilized for carotenoid production as described hereinafter.

An organism capable of carotenoid synthesis and a potential source of genes for such an endeavor is Erwinia herbicola, which is believed to carry putative genes for carotenoid production on a plasmid (Thiry, J. Gen. Microbiol., 130:1623 (1984)) or chromosomally (Perry et al., J. Bacteriol., 168:607 (1986)). Erwinia herbicola is a genus of Gram-negative bacteria of the ENTEROBACTERIACEAE family, which are facultative anaerobes. Indeed, recently published European patent application 0 393 690 A1 (published April 20, 1990; sometimes referred to herein as "EP 0 393 690") reports use of DNA from Erwinia uredovora 20D3 (ATCC 19321) for preparing carotenoid molecules.

As is discussed in detail hereinafter, the present invention utilizes DNA from Erwinia herbicola EHO-10 (AT 39368) for preparation of carotenoid molecules and the enzymes used in their synthesis. Erwinia herbicola EHO-10 used herein is also referred to as Escherichia vulneris.

-6-

The genus is commonly divided into three groups. Of the three, the *Herbicola* group includes species (e.g. *E. herbicola*) which typically form yellow pigments that have now been found to be carotenoids.

5 These bacteria exist as saprotrophs on plant surfaces and as secondary organisms in lesions caused by many plant pathogens. They can also be found in soil, water and as opportunistic pathogens in animals, including man.

10 A precise organismic function has yet to be ascribed to the pigment(s) produced by *Erwinia herbicola*. Perry et al., *J. Bacteriol.*, 168:607 (1986), showed that the genes coding for the production of a then unknown yellow pigment lie within an
15 approximately 13-kilobase (kb) sequence coding for at least seven polypeptides, and that the expression of the yellow pigment is cyclic AMP mediated. Tuveson, *J. Bacteriol.*, 170:4675 (1988), demonstrated that these
20 genes, cloned from *Erwinia herbicola* and expressed in an *E. coli* strain, offered the host some protection against inactivation by near-UV light and specific phototoxic molecules.

E. coli and *S. cerevisiae* are commonly used for expressing foreign genes, but to optimize yields
25 and minimize technical maintenance procedures, it would be preferable to utilize a higher plant species.

Brief Summary of the Invention

30 One aspect contemplated by this invention is an isolated DNA segment comprising a nucleotide sequence that defines a structural gene or variant DNA thereof capable of expressing each of the *Erwinia herbicola* genes for GGPP synthase (including a DNA analog), phytoene synthase, phytoene dehydrogenase-4H,

-7-

lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase in biologically active form.

Another aspect of this invention is a recombinant DNA molecule comprising a vector
5 operatively linked to an above exogenous DNA segment isolated from Erwinia herbicola or a variant DNA. This exogenous DNA segment defines a structural gene capable of expressing any one of the above Erwinia herbicola enzymes. Also included is a promoter suitable for
10 driving the expression of the enzyme in a compatible host organism. Exemplary, particularly preferred vectors include plasmids pARC417BH, pARC489B, pARC489D, pARC285, pARC140N, pARC145G, pARC496A, pARC146D, pATC228, pATC1616, pARC1509, pARC1510, pARC1520,
15 pARC404BH, pARC406BH, pARC145H and pARC2019.

A further aspect of this invention is a method for preparing each of the above-mentioned E. herbicola enzymes. This method comprises initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host
20 cells transformed with a recombinant DNA molecule containing an expression vector compatible with the cells. This vector is operatively linked to an isolated exogenous Erwinia herbicola DNA segment or variant DNA that defines the structural gene for an
25 above-mentioned particular enzyme. The culture is maintained for a time period sufficient for the cells to express the enzyme.

Still another aspect contemplated by this invention is a method for producing GGPP, phytoene,
30 lycopene, β -carotene, zeaxanthin and/or zeaxanthin diglucoside that comprises initiating a culture in a nutrient medium of prokaryotic or eukaryotic host cells that provides the immediate precursor to the desired product, those prokaryotic or eukaryotic host cells
35 being transformed with one or more recombinant DNA

molecule(s) described herein that include a structural gene that can express an enzyme that converts the precursor to the product desired. The culture is maintained for a time period sufficient for the host cells to express the enzyme and for the expressed enzyme to convert the provided precursor into product. A recombinant DNA molecule contains an expression vector compatible with the host cells operatively linked to one or more exogenous Erwinia herbicola DNA or variant DNA segments comprising (i) a nucleotide base sequence corresponding to a sequence defining a structural gene for geranylgeranyl pyrophosphate synthase, (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase, (iii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene dehydrogenase-4H, (iv) a nucleotide base sequence corresponding to a sequence defining a structural gene for lycopene cyclase, (v) a nucleotide base sequence corresponding to a sequence defining a structural gene for beta-carotene hydroxylase, and/or (vi) a nucleotide base sequence corresponding to a sequence defining a structural gene for zeaxanthin glycosylase as previously described. The culture is maintained for a time period sufficient for the cells to express the enzyme products of the desired structural genes (i), (ii), (iii), (iv), (v) and/or (vi), and form a product that is desired.

Yet another aspect of this invention is a method of protecting a higher plant from the herbicide norflurazon. Here, a higher plant to be protected is transformed with a recombinant DNA molecule that encodes a structural gene for the Erwinia herbicola enzyme phytoene dehydrogenase-4H or a DNA variant thereof that encodes an enzyme exhibiting substantially

the same biological activity. The transformed plant is maintained for a time period sufficient for phytoene dehydrogenase-4H to be expressed, and transformed plant is treated with a herbicidal amount of norflurazon.

5 In particularly preferred practice, all of the recombinant DNA utilized in this invention is from Erwinia herbicola. Another preferred embodiment of this invention is a recombinant DNA molecule as described above, wherein the promoter is Rec 7 for
10 E. coli, PGK, GAL 10 and GAL 1 for yeasts such as S. cerevisiae and CaMV 35S for higher plants.

Other preferred embodiments contemplate the methods of preparation described above, wherein the host transformed is either a prokaryote, such as
15 E. coli, a eukaryote, for example yeast such as S. cerevisiae, or a higher plant, such as alfalfa or tobacco. Because of the utility of GGPP and the carotenoids as chemical precursors and as effective and apparently harmless food colorants, the ability to
20 produce these materials in commercially advantageous amounts from transgenic biological sources with the aid of recombinant DNA technology is a major benefit flowing from this invention. To realize these
25 benefits, the above aspects and embodiments are contemplated by this invention. Still further embodiments and advantages of the invention will become apparent to those skilled in the art upon reading the entire disclosure contained herein.

30 Brief Description of Drawings

Figure 1 is a flow diagram of the carotenoid biosynthesis pathway utilizing the Erwinia herbicola gene complement located in the plasmid pARC376.

Figure 2 in three sheets as **Figure 2-1**, **Figure**
35 **2-2**, and **Figure 2-3** illustrates the nucleotide base

-10-

sequences of certain preferred DNA segments of the structural gene for geranylgeranyl pyrophosphate (GGPP) synthase (SEQ ID NO: 1). The base sequences are shown conventionally from left to right and in the direction of 5' terminus to 3' terminus, using the single letter nucleotide base code.

The reading frame of the 5' end of the structural gene illustrated herein is indicated by placement of the deduced, amino acid residue sequence (SEQ ID NO: 2) of the protein for which it codes below the nucleotide sequence, such that the triple letter code for each amino acid residue is located directly below the three-base codon for each amino acid residue. Numerals to the right of the DNA sequence indicate nucleotide base positions within the DNA sequence shown. All of the structural genes shown in the figures herein are similarly illustrated, with amino acid initiation position beginning here with the initial methionine residue (Met) at DNA position about 124 as shown.

Several restriction enzyme sites of importance are indicated above the DNA sequence. These represent points of manipulation in engineering the gene construct encoding the enzyme.

Figure 3 shown in three sheets as Figure 3-1, Figure 3-2 and Figure 3-3 illustrates the DNA (SEQ ID NO: 3) and deduced amino acid residue (SEQ ID NO: 4) sequences of more preferred, heterologous structural genes of Erwinia herbicola GGPP synthase. Here, the expressed protein begins with the Met residue at about position 150 as shown and terminates within the Eco RV site (about 1153) in the DNA construct present in plasmid pARC489B, whereas the gene terminates at the Bal I site (about 1002) in the DNA construct present in plasmid pARC489D. The short amino-terminal sequence

-11-

MetAlaGluPhe (about 150-161) is a heterologous sequence from plasmid pARC306A, and is substituted for the native sequence from DNA position 124 to 150 shown in Figure 2.

5 Figure 4 shown in three sheets as Figure 4-1, Figure 4-2 and Figure 4-3 illustrates the nucleotide (SEQ ID NO: 5) and amino acid (SEQ ID NO: 6) sequences of the structural gene for phytoene synthase.

10 The Met initiation codon (about position 16 as shown) corresponds to about position 6383 on pARC376 in Figure 5. The Bam HI restriction site at about 1093 in Figure 4 corresponds to the Bam HI site at about position 5302 on pARC376 in Figure 5. The illustrated Bgl II restriction site shown at about position 8 is
15 not present in the native DNA sequence and was added as is discussed hereinafter.

 Figure 5 schematically illustrates the plasmid pARC376 containing the full complement of enzyme genes, represented by capital letters, required for the
20 synthesis of carotenoids from farnesyl pyrophosphate, as indicated in the schematic of Figure 1. Note that the direction of transcription (arrows) is uniform for all enzyme structural genes except beta-carotene hydroxylase (F), which is transcribed in an opposite
25 direction. Important restriction enzyme sites are also identified. The synthesis of phytoene is catalyzed by the enzymes GGPP synthase (A) and phytoene synthase (E). The gene labeled D encodes the enzyme phytoene dehydrogenase-4H. Genes labeled B, C and F encode the
30 enzymes zeaxanthin glycosylase, lycopene cyclase, and beta-carotene hydroxylase, respectively. The overlap of genes E and F is shown by hatching.

 Figure 6 is a schematic representation of the plasmid pARC306A, which contains the Rec 7 promoter.
35 This plasmid also has multiple cloning sites adjacent

to the Rec 7 promoter and 5' and 3' transcription termination loops. Approximate positions of restriction enzyme sites are shown.

5 Figure 7 illustrates schematically the plasmid pARC135, which contains the S. cerevisiae phosphoglyceric acid kinase (PGK) promoter operatively linked at the Bgl II site. Various additional features of the plasmid are also illustrated.

10 Figure 8 shows a schematic representation of the vector pSOC713, including a partial restriction enzyme map.

15 Figure 9 is a schematic representation of plasmid pARC145B, which is a yeast/E. coli shuttle vector for expression of introduced genes in yeast, including a partial restriction enzyme map.

20 Figure 10 is a schematic representation of the vector pARC145G, which is basically pARC145B above that contains the two preferred genes; i.e., GGPP synthase and phytoene synthase, each operatively linked at their 5' ends to the divergent promoters GAL 10 and GAL 1. Phytoene synthase also has a PGK terminator at the 3' end.

25 Figure 11 shown in four panels as Figure 11-1, Figure 11-2, Figure 11-3 and Figure 11-4 illustrates the DNA (SEQ ID NO: 7) and deduced amino acid residue (SEQ ID NO: 8) sequences of the Erwinia herbicola structural gene for phytoene dehydrogenase-4H. The Met codon (shown at position 7) corresponds to position 7849 on plasmid pARC376 in Figure 5.

30 Figure 12 is a schematic representation of the vector pSOC925, including a partial restriction enzyme map.

35 Figure 13 is a schematic representation of plasmid pARC146, including a partial restriction enzyme map.

-13-

Figure 14 shows the vector pARC146D, including a partial restriction enzyme map.

Figure 15 shown in four panels as Figures 15-1, Figures 15-2, Figure 15-3 and Figure 15-4 illustrates the DNA (SEQ ID NO: 9) and deduced amino acid residue (SEQ ID NO: 99) sequence of the Erwinia herbicola structural gene for phytoene dehydrogenase-4H present in plasmid pARC146D.

Figure 16 is a schematic representation of plasmid pATC228, including a partial restriction enzyme map. In this figure, A-F are schematic representations of the following sequences: A=tac promoter, B=phytoene dehydrogenase-4H gene, C=pMB1 ori, D=ampicillin resistance gene, E=chloramphenicol resistance gene, and F=R1162 ori.

Figure 17 illustrates the coding sequence and encoded transit peptide (SEQ ID NO: 98) DNA (SEQ ID NO: 10) sequence linked to the 5' end of the phytoene dehydrogenase-4H structural gene for transport of the phytoene dehydrogenase-4H enzyme into tobacco chloroplasts as well as other plant chloroplasts. Stars over nucleotide positions 69 and 72 in this sequence indicate G for T and G for A replacements utilized to introduce an Nar I site.

Figure 18 is a schematic representation of the about 15.6 kb plasmid pATC1616, including a partial restriction enzyme map. In this figure, A-I are schematic representations of the following sequences: A=CaMV 35S promoter, B=transit peptide sequence, C=phytoene dehydrogenase-4H gene, D=NOS polyadenylation site, E=pBR322 ori, F=ori T, G=tetracycline resistance gene, H=ori V, and I=kanamycin resistance gene.

Figure 19 shown in three panels as Figure 19-1, Figure 19-2 and Figure 19-3 illustrates the DNA (SEQ ID NO: 11) and a deduced amino acid residue (SEQ ID NO:

-14-

13) sequences of the Erwinia herbicola structural gene for lycopene cyclase.

The Met codon (shown at position 19) corresponds to position 9002 on plasmid pARC376 in Figure 5. The restriction sites Sph I and Bam HI were introduced at the 5' and 3' ends of the gene using PCR. The changes in the sequence for the genetically engineered version of the gene (SEQ ID NO: 12) used for expression in yeast are shown in bold underneath the native sequence. At the 5' end of the gene, the native initiation GTG codon has been changed to an ATG codon. The second amino acid residue, Arg, was originally encoded by an AGG codon that was changed to a CGG codon, while retaining its coding for the Arg amino acid residue.

Figure 20 shown in three panels as Figure 20-1, Figure 20-2 and Figure 20-3 illustrates the DNA sequence of the native Erwinia herbicola DNA (SEQ ID NO: 14) segment containing the structural gene for beta-carotene hydroxylase, corresponding to a DNA segment from position 4886 to position 5861 of pARC376 shown in Figure 5. The Met initiation codon is located at position 25 as shown, which corresponds to position 4991 in Figure 5.

Figure 21 shown in three panels as Figure 21-1, Figure 21-2 and Figure 21-3 illustrates the DNA (SEQ ID NO: 15) and deduced amino acid residue (SEQ ID NO: 16) sequences of the engineered Erwinia herbicola structural gene for beta-carotene hydroxylase. The Met codon (shown at position 25) corresponds to position 4991 on plasmid pARC376 in Figure 5. The restriction sites Nco I and Sma I were introduced at the 5' and 3' ends of the gene as described in Example 21. The changes in the sequence for the genetically engineered version of the gene are shown in bold. At the 5' end

-15-

of the gene, the native second and third amino acid residues have been changed from -Leu-Val- to -Val-Leu-.

Figure 22 is a schematic representation of plasmid pARC300E, including a partial restriction enzyme map showing restriction sites present only once.

Figure 23 is a schematic representation of plasmid pARC300M, including a partial restriction enzyme map showing restriction sites present only once.

Figure 24 is a schematic representation of plasmid pARC300T, including a partial restriction enzyme map showing restriction sites present only once.

Figure 25 shown in three panels as Figure 25-1, Figure 25-2 and Figure 25-3 illustrates the DNA (SEQ ID NO: 97) and deduced amino acid residue (SEQ ID NO: 17) sequences of the engineered Erwinia herbicola structural gene for zeaxanthin glycosylase.

Detailed Description of the Invention

A. Definition of Terms

Amino Acid: All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-59, (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

-16-

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>			<u>AMINO ACID</u>
	<u>1-Letter</u>	<u>3-Letter</u>	
5	Y	Tyr	L-tyrosine
	G	Gly	glycine
	F	Phe	L-phenylalanine
	M	Met	L-methionine
10	A	Ala	L-alanine
	S	Ser	L-serine
	I	Ile	L-isoleucine
	L	Leu	L-leucine
	T	Thr	L-threonine
15	V	Val	L-valine
	P	Pro	L-proline
	K	Lys	L-lysine
	H	His	L-histidine
	Q	Gln	L-glutamine
20	E	Glu	L-glutamic acid
	W	Trp	L-tryptophan
	R	Arg	L-arginine
	D	Asp	L-aspartic acid
	N	Asn	L-asparagine
25	C	Cys	L-cysteine

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

Expression: The combination of intracellular processes, including transcription and translation undergone by a structural gene to produce a polypeptide.

-17-

Expression vector: A DNA sequence that forms control elements that regulate expression of structural genes when operatively linked to those genes.

Operatively linked or inserted: A structural gene is covalently bonded in correct reading frame to another DNA (or RNA as appropriate) segment, such as to an expression vector so that the structural gene is under the control of the expression vector.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Recombinant DNA molecule: A hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

Structural gene: A DNA sequence that is expressed as a polypeptide, i.e., an amino acid residue sequence.

Vector: A DNA molecule capable of replication in a cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

25

B. Introduction

Constituting the most widespread group of pigments, carotenoids are present in all photosynthetic organisms, where they are an essential part of the photosynthetic apparatus.

Mevalonic acid, the first specific precursor of all the terpenoids is formed from acetyl-CoA via HMG-CoA (3-hydroxy-3-methylglutaryl-CoA), and is itself converted to isopentenyl pyrophosphate (IPP), the universal isoprene unit. After isomerization of IPP to

35

-18-

dimethylallyl pyrophosphate and a series of
condensation reactions adding IPP, geranylgeranyl
pyrophosphate (GGPP) is formed according to the scheme
in Figure 1. The formation of GGPP is the first step
5 in carotenoid biosynthesis.

In the bacterium Erwinia herbicola, phytoene
has now been found to be formed biosynthetically in a
two-step process as shown in Figure 1. The initial
step is the condensation of farnesyl pyrophosphate
10 (FPP) and isopentenyl pyrophosphate (IPP) to form
geranylgeranyl pyrophosphate (GGPP). This reaction is
catalyzed by the enzyme geranylgeranyl pyrophosphate
synthase (GGPP synthase). This first step is
immediately followed by a tail-to-tail dimerization of
15 GGPP, catalyzed by the enzyme phytoene synthase, to
form phytoene. This pathway thus differs from the
pathway reported in published European Application
0 393 690 wherein GGPP is said to form prephytoene
pyrophosphate (a cyclopropylene-containing molecule)
20 that thereafter forms phytoene.

Lycopene has now been found to be the second
carotenoid produced in Erwinia herbicola. The third
carotenoid produced by Erwinia herbicola results from
the cyclization of lycopene to form beta-carotene, by
25 the enzyme lycopene cyclase. The fourth carotenoid in
the Erwinia herbicola pathway is zeaxanthin that is
produced from beta-carotene. The fifth carotenoid,
zeaxanthin diglucoside, is produced from zeaxanthin.

The present invention relates to these steps
30 in the carotenoid biosynthesis pathway, the methods of
isolating the Erwinia herbicola genes encoding
carotenoid biosynthesis enzymes of the pathway and to
the adaptation of this pathway by recombinant DNA
technology to achieve methods and capabilities of GGPP
35 and carotenoid production, particularly in host

-19-

organisms that do not otherwise synthesize those materials, but in relatively small amounts or in specialized locations.

5 The disclosure below provides a detailed description of the isolation of carotenoid synthesis genes from Erwinia herbicola, modification of these genes by genetic engineering, and their insertion into compatible plasmids suitable for cloning and expression in E. coli, yeasts, fungi and higher plants. Also
10 disclosed are methods for preparation of the appropriate enzymes and the methods for GGPP and carotenoid production in these various hosts.

Plasmid constructs are exemplified for several host systems. However, similar constructs utilizing
15 the genes of this invention are available for virtually any host system as is well known in the art.

A structural gene or isolated, purified DNA segment of this invention is often referred to as a restriction fragment bounded by two restriction
20 endonuclease sites and containing a recited number of base pairs. A structural gene of this invention is also defined to include a sequence shown in a figure plus variants of such genes (described hereinafter), that hybridize non-randomly with a gene shown in the
25 figure under stringency conditions as described hereinafter. Each contemplated gene includes a recited non-randomly-hybridizable variant DNA sequence, encodes a particular enzyme and also produces biologically active molecules of the encoded enzymes when suitably
30 transfected into and expressed in an appropriate host.

Polynucleotide hybridization is a function of sequence identity (homology), G+C content of the sequence, buffer salt content, sequence length and duplex melt temperature (T_m) among other variables.
35 See, Maniatis et al., Molecular Cloning, Cold Spring

-20-

Harbor Laboratory, Cold Spring Harbor, N.Y. (1982),
page 388.

5 With similar sequence lengths, the buffer salt
concentration and temperature provide useful variables
for assessing sequence identity (homology) by
hybridization techniques. For example, where there is
at least 90 percent homology, hybridization is carried
out at 68°C in a buffer salt such as 6XSSC diluted from
20XSSC [Maniatis et al., above, at page 447]. The
10 buffer salt utilized for final Southern blot washes can
be used at a low concentration, e.g., 0.1XSSC and at a
relatively high temperature, e.g. 68°C, and two
sequences will form a hybrid duplex (hybridize). Use
of the above hybridization and washing conditions
15 together are defined as conditions of high stringency
or highly stringent conditions.

Moderately high stringency conditions can be
utilized for hybridization where two sequences share at
least about 80 percent homology. Here, hybridization
20 is carried out using 6XSSC at a temperature of about
50-55°C. A final wash salt concentration of about
1-3XSSC and at a temperature of about 60-68°C are used.
These hybridization and washing conditions define
moderately high stringency conditions.

25 Low stringency conditions can be utilized for
hybridization where two sequences share at least 40
percent homology. Here, hybridization carried out
using 6XSSC at a temperature of about 40-50°C, and a
final wash buffer salt concentration of about 6XSSC
30 used at a temperature of about 40-60°C effect non-
random hybridization. These hybridization and washing
conditions define low stringency conditions.

An isolated DNA or RNA segment that contains a
nucleotide sequence that is at least 80 percent, and
35 more preferably at least 90 percent identical to a DNA

-21-

sequence for gene shown in a figure is contemplated by this invention. Such a nucleotide sequence, when present in a host cell as part of a plasmid or integrated into the host genome as described herein, that also hybridizes non-randomly under at least moderately high stringency conditions, and encodes and expresses a biologically active enzyme is contemplated herein as a variant DNA of an illustrated sequence that exhibits substantially the same biological activity.

In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid sequence of the structural gene that codes for the protein. Thus, a structural gene can be defined in terms of the amino acid residue sequence; i.e., protein or polypeptide, for which it codes.

Thus, through the well-known redundancy of the genetic code, additional DNA and corresponding RNA sequences can be prepared that encode the same amino acid residue sequences, but that are sufficiently different from a before-discussed gene sequence that the two sequences do not hybridize at high stringency, but do hybridize at moderately high stringency. Furthermore, allelic variants of a structural gene can exist in other Erwinia herbicola strains that are also useful, but form hybrid duplex molecules only at moderately high stringency.

A DNA or RNA sequence that (1) encodes an enzyme molecule exhibiting substantially the same biological activity as an enzyme molecule expressed by a DNA sequence of a figure, (2) hybridizes with a DNA sequence of one of those figures at least at moderately high stringency and (3) shares at least 80 percent, and more preferably at least 90 percent, identity with a DNA sequence of those figures is defined as a variant

-22-

DNA sequence. Thus, a DNA variant or variant DNA sequence is defined as including an RNA sequence.

In comparing DNA sequences of E. herbicola and E. uredovora, the published European Application 0 393 690 reported no hybridization of a DNA probe from E. uredovora with DNA from E. herbicola using highly stringent hybridization conditions. Present studies indicate a range of sequence identities of about 55 to about 70 percent between the sequences of that published European application and the sequences disclosed herein, with there being about a 65 percent identity between the two genes for beta-carotene hydroxylase. In view of the 45 to 30 percent of mismatched base pairs, and the reported non-hybridization at high stringency of the herbicola and uredovora DNAs, the reported E. uredovora DNA sequences and the E. herbicola DNAs discussed herein are distinguishable, and genes that produce similar enzymes are not variants.

Variant DNA molecules that encode and can express a desired GGPP or carotenoid enzyme can be obtained from other organisms using hybridization and functionality selection criteria discussed herein. For example, a microorganism, fungus, alga, or higher plant that is known or can be shown to produce a carotenoid is utilized as a DNA source. The total DNA of the selected organism is obtained and a genomic library is constructed in a λ phage such as λ gt11 using the protocols discussed in Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982) at pages 270-294.

The phage library is then screened under standard protocols using a radiolabeled, nick-translated DNA probe having a sequence of the Erwinia herbicola DNA of the figures, and the before-discussed

-23-

moderate or high stringency hybridization conditions. Once the hybridization studies locate the appropriate structural gene, that structural gene DNA segment can be obtained, sequenced, engineered for expression in an appropriate recombinant molecule and shown to produce a biologically active enzyme as is discussed elsewhere herein and other techniques and protocols that are well known to workers skilled in molecular biology.

That a DNA sequence variant encodes a "biologically active" enzyme or an enzyme that has "substantially the same biological activity" is determined by whether the variant DNA sequence produces an enzyme as discussed herein. Thus, a DNA variant sequence that expresses GGPP synthase or a carotenoid biosynthesis enzyme that converts a provided precursor substrate molecule into a desired product is defined as biologically active. Expression of a biologically active enzyme from a variant DNA sequence can be assayed by the production of the desired product.

A DNA segment of the invention thus includes a DNA sequence that encodes Erwinia herbicola GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase beta-carotene hydroxylase or zeaxanthin glycosylase of a figure, or a variant DNA. In a preferred embodiment, that DNA segment includes a DNA sequence that encodes the enzyme in a DNA segment separate from any other carotenoid-forming enzyme encoding sequences. More preferably, a DNA segment contains the Erwinia herbicola GGPP synthase or carotenoid biosynthesis enzyme structural gene, and is free from a functional gene whose expression product consumes the desired carotenoid, or otherwise inhibits carotenoid production.

-24-

C. Genes Encoding Enzymes for GGPP and Carotenoid Biosynthesis

1. Isolation of the carotenoid gene cluster

The plasmid pARC376 contains an approximately 13 kb chromosomal DNA fragment isolated by Perry et al. J. Bacteriol., 168:607 (1986) from the bacterium Erwinia herbicola EHO-10 (Escherichia vulneris; ATCC 39368) that when transferred into the bacterium E. coli causes the E. coli cells to produce a yellow pigment. Plasmid pARC376 was referred to by those authors as plasmid pPL376. A restriction map of the pARC376 plasmid is shown in Figure 5.

The structural genes in the plasmid responsible for pigment production are present on a DNA fragment of about 7900 base pairs (bp) that is bounded by the restriction sites Pst I (at about position 4886) and Bgl II (at about position 12349) shown in Figure 5. There are a total of six relevant genes in this approximately 7900 bp region that cause the E. coli cells to produce GGPP and the carotenoids phytoene through zeaxanthin diglucoside, which is the final product identified in the carotenoid pathway contained in plasmid pARC376.

The biosynthetic pathway for carotenoid production through zeaxanthin diglucoside is shown in Figure 1. E. coli cells, and all cells contemplated as hosts herein, naturally synthesize the isoprenoid intermediate farnesyl pyrophosphate (FPP). The genes for geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and zeaxanthin glycosylase are located in the approximately 7900 bp DNA fragment in pARC376. E. coli cells that are transformed with the plasmid pARC376 are able to

-25-

convert some of the endogenous FPP into carotenoids by utilizing the enzymes encoded on the plasmid.

The following are descriptions of the individual structural genes, including the genes of this invention responsible for the synthesis of GGPP and carotenoids, and the recombinant DNA manipulations that have been performed to influence carotenoid biosynthesis in bacteria such as E. coli, yeast such as S. cerevisiae and higher plants.

2. GGPP Synthase Gene and Plasmid Constructs

a. DNA segments

An isolated, purified DNA segment comprising a nucleotide sequence of at least 850 base pairs that define a structural gene for the Erwinia herbicola enzyme GGPP synthase and its DNA variants is one structural gene contemplated by this invention. A typical, useful DNA segment contains about 850 to about 1150 base pairs, whereas a more preferred DNA segment contains about 850 to about 1000 base pairs. The native sequence includes about 924 bp. Larger DNA segments are also contemplated and are discussed hereinafter.

An approximately 1153 bp fragment that extends from the Bgl II (about 12349) site to the Eco RV (about 11196) site of plasmid pARC376 is shown in Figure 5. A preferred structural gene for GGPP synthase is within the about 1153 bp Bgl II to Eco RV restriction fragment shown in Figure 5 and contains the previously mentioned native structural gene of about 924 bp. This structural gene is within the approximately 1030 bp Nco I-Eco RV restriction fragment of plasmid pARC417BH.

Surprisingly it has been found that a recombinant structural gene that encodes an amino-terminal truncated version of this enzyme in which the

amino-terminal thirteen residues of the native enzyme were deleted and were replaced by four extraneous amino acid residues from the pARC306A vector was more active (about two times) than was a recombinantly produced enzyme having the encoded, native thirteen amino-terminal residues. This more active enzyme is encoded by the structural GGPP synthase gene containing about 1000 bp shown in Figure 3, and is within the approximately 1150 bp segment Nco I-Pvu II restriction fragment of plasmid pARC489B.

Still more surprisingly, it has also been found that truncation of the carboxy-terminus of the GGPP synthase molecule made the enzyme still more active. Thus, use of a GGPP synthase structural gene of Figure 3 from which the 3' Bal I-Eco RV fragment was removed provided the most active GGPP synthase found. This structural gene of about 850 bp is within the approximately 1000 bp Nco I-Pvu II restriction fragment of pARC489D. This GGPP synthase gene is most preferred herein. Details of the above work are described hereinafter.

The DNA sequence 1 from E. uredovora in EP 0 393 690 is said there to encode the gene for converting prephytoene pyrophosphate to phytoene. The DNA sequence of that European application has about 59 percent identity with the GGPP synthase illustrated herein. That E. uredovora DNA sequence 1 is an analog of the before-discussed GGPP synthase gene, and can also be used herein for preparing GGPP.

b. Recombinant DNA molecules

Also useful in this invention, are recombinant DNA molecules comprising a vector operatively linked to an exogenous DNA segment defining a structural gene capable of expressing the enzyme GGPP synthase, as

-27-

described above, and a promoter suitable for driving the expression of the gene encoding the enzyme in a compatible host organism. The vector and promoter are as described elsewhere herein. Particularly preferred
5 plasmid vectors include pARC417BH, pARC489B, pARC489D and pARC145G.

3. Phytoene Synthase Gene and Plasmid Construct

a. DNA segments

10 An isolated, purified DNA segment comprising a nucleotide sequence of at least about 927 base pairs that define a structural gene for the Erwinia herbicola enzyme phytoene synthase and its DNA variants is also contemplated in this invention by producing phytoene
15 from GGPP. This structural gene typically contains about 1000 to about 1250 bp including the 927 bp of the native sequence, but can also contain a greater number as discussed hereinafter. The structural gene for phytoene synthase lies between positions 6383 and 5457
20 of plasmid pARC376 (Figure 5).

A phytoene synthase gene useful herein at least includes a sequence shown in Figure 4. In preferred practice, the structural gene also includes an upstream sequence shown in Figure 4 from about
25 position 8 (Bgl II site) to about position 15 (Nco I site).

A preferred phytoene synthase gene is within the about 1112 bp Nco I-Eco RI fragment of plasmid pARC285. Also included within that about 1112 bp
30 segment is the approximately 1040 bp Nco I-Bam HI fragment that also encodes the desired structural gene.

The most preferred structural gene includes a nucleotide base sequence in Figure 4 from about base 8 to about base 15 as well as from about base 841 to
35 about base 1040, and contains about 1090 bp. This most

-28-

preferred gene is contained in the approximately 1176 base pair sequence of the Hpa I to Bam HI restriction sites and approximately 1238 bp Pvu II-Eco RI fragments present in the plasmid pARC140N, as well as in the
5 approximately 1088 bp sequence of the Bgl II-Eco RI fragment of plasmid pARC140R.

b. Recombinant DNA molecules

A recombinant DNA molecule, comprising a
10 vector operatively linked to an exogenous DNA segment defining a structural gene capable of expressing the enzyme phytoene synthase, as discussed above, and a promoter suitable for driving the expression of the gene in a compatible host organism, is also useful in
15 this invention. The vector and promoter of this recombinant molecule are also as are discussed herein. Particularly preferred plasmid vectors include pARC285, pARC140N, and pARC145G.

20 **4. Phytoene Dehydrogenase-4H Gene and Plasmid Construct**

a. DNA Segment

Another DNA segment of this invention is an isolated DNA segment comprising a nucleotide sequence
25 that contains at least about 1470 bp that includes a sequence defining a structural gene capable of expressing the Erwinia herbicola enzyme phytoene dehydrogenase-4H and its DNA variants. Phytoene dehydrogenase-4H converts phytoene to lycopene. This
30 phytoene dehydrogenase-4H enzyme has a molecular mass of about 51,000 daltons. The native phytoene dehydrogenase-4H structural gene contains about 1470 bp and is located between positions 7849 and 6380 of plasmid pARC376.

A typical, useful DNA segment contains about 1500 base pairs and lies within the approximately 1891 bp Ava I (8231) to Nco I (6342) DNA fragment from pARC376 illustrated in Figure 5. Larger DNA segments are also contemplated, as discussed hereinafter.

A preferred DNA segment includes a nucleotide base sequence shown in Figure 11 from about base 15 to about base 1470. Particularly preferred DNA segments include the bases between the engineered Nco I site at about position 5 of Figure 11-1 (the initial Met residue) and about position 1470 of Figure 11-4, and is present in the approximately 1505 bp Nco I-Nco I restriction fragment (Nco I fragment) of plasmid pARC496A, the approximately 1508 bp Sal I-Sal I restriction fragment (Sal I fragment) of plasmid pARC146D, and the approximately 1506 bp Sph I-Nco I fragment present in plasmid pATC228. The sequence of the about 1508 bp Sal I fragment is illustrated in Figure 15.

A still further particularly preferred DNA segment is the approximately 2450 bp Xba I-Xba I fragment present in plasmid pATC1616. This fragment contains an approximately 1683 bp portion that encodes a chloroplast transit peptide of tobacco ribulose bisphosphate carboxylase-oxygenase (hereinafter referred to as a chloroplast transit peptide) (about 177 bp) operatively linked in frame to the 5' end of the above Sph I-Nco I about 1506 bp phytoene dehydrogenase-4H gene. That approximately 1683 bp fragment is flanked at its 5' end by an about 450 bp CaMV 35S promoter sequence and at its 3' end by an about 300 bp NOS polyadenylation sequence.

This DNA segment can be used for expression of phytoene dehydrogenase-4H in higher plants and transport of the expressed phytoene dehydrogenase-4H

into chloroplasts such as those of tobacco. Infection of a higher plant such as tobacco with A. tumefaciens containing plasmid pATC1616 caused genomic incorporation of DNA for the promoter, transit peptide-phytoene dehydrogenase-4H and NOS sequence, and makes the resultant plants resistant to the herbicide norflurazon.

It is noted that restriction fragments having the same restriction enzyme cleavage sequence at both the 5' and 3' ends are sometimes referred to herein by reference to a single restriction enzyme. Thus, the approximately 1505 bp Nco I-Nco I restriction fragment referred to above can also be referred to herein as an approximately 1505 bp Nco I fragment. Similarly, the approximately 1508 bp Sal I-Sal I fragment can be referred to as the approximately 1508 bp Sal I fragment, and the approximately 2450 bp Xba I-Xba I fragment can be referred to as the approximately 2450 bp Xba I fragment.

b. Recombinant DNA Molecules

A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment defining a structural gene capable of expressing the enzyme phytoene dehydrogenase-4H and a promoter suitable for driving the expression of the enzyme in a compatible host organism is also contemplated by this invention. The structural gene has a nucleotide base sequence described above. Particularly preferred plasmids include pARC496A, pARC146D, pATC228 and pATC1616.

5. Lycopene Cyclase Gene and Plasmid Construct

a. DNA Segment

Also contemplated by this invention is an isolated DNA segment comprising a nucleotide sequence

-31-

that contains at least about 1125 base pairs that includes a sequence defining a structural gene capable of expressing the Erwinia herbicola enzyme lycopene cyclase and its DNA variants. This lycopene cyclase enzyme has a molecular mass of about 39,000 daltons and converts lycopene to β -carotene. A typical, useful DNA segment contains at least about 1125 base pairs and preferably at least about 1150 base pairs and lies within the approximately 1548 bp Sal I (9340) to Pst I (7792) DNA fragment from plasmid pARC376 illustrated in Figure 5. The native Erwinia herbicola structural gene for lycopene cyclase contains about 1125 base pairs and is located between positions 9002 and 7878 of plasmid pARC376. Larger DNA segments are also contemplated, as discussed hereinafter.

A preferred DNA segment includes a nucleotide base sequence shown in Figure 19, panels 1 and 2, from about base 1 to about base 1222. A more preferred sequence of about 1140 bp is present in the approximately 1142 bp Sph I-Bam HI restriction fragment of the plasmid pARC1509, and shown in Figure 19.

A still further particularly preferred DNA segment is an approximately 1319 bp Nco I-Bam HI fragment. This fragment contains an approximately 177 bp portion that encodes a chloroplast transit peptide operatively linked in frame to the 5' end of the above Sph I-Bam HI 1142 bp lycopene cyclase gene. This DNA segment can be used for expression of lycopene cyclase in higher plants and transport of the expressed lycopene cyclase into chloroplasts such as those of tobacco.

b. Recombinant DNA Molecules

A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment defining

-32-

a structural gene capable of expressing the enzyme lycopene cyclase and a promoter suitable for driving the expression of that enzyme in a compatible host organism, is also contemplated by this invention. The structural gene has a nucleotide base sequence described above. Particularly preferred plasmid vectors include pARC1510, pARC1520, pARC1509.

6. Beta-Carotene Hydroxylase Gene and Plasmid Construct

a. DNA Segment

Further contemplated by this invention is an isolated DNA segment comprising a nucleotide sequence that contains at least about 531 base pairs and more preferably about 878 base pairs, including a sequence defining a structural gene capable of expressing the Erwinia herbicola enzyme beta-carotene hydroxylase, an enzyme that synthesizes zeaxanthin, and DNA variants of that gene. The native enzyme is encoded between positions 4991 and 5521 of plasmid pARC376.

One nucleotide base sequence corresponds to the sequence in Figure 20 from about base 1 to about base 894 at the Sma I site, and preferably from about base 1 to about base 752. More preferably, the DNA segment utilized is that shown in Figure 21 from about base 25 to about base 897. The latter sequence constitutes the about 870 bp Nco I to Sma I DNA fragment contained in plasmid pARC406BH. A contemplated DNA segment lies within the (4991) to (5861) DNA segment of about 870 base pairs of plasmid pARC376 illustrated in Figure 5.

A still further particularly preferred DNA segment is an Xba I-Xba I fragment including about 1797 bp constituted by the following sequence of genes: (a) the about 450 bp CaMV 35S promoter, (b) the 177 bp

-33-

sequence that encodes a chloroplast transit peptide operatively linked in frame to the 5' end of (c) the about 870 bp beta-carotene hydroxylase gene, and (d) the about 300 bp NOS polyadenylation sequence. This
5 Xba I fragment can be cloned into the Xba I site of plasmid pGA482, with the resulting plasmid being used to transform A. tumefaciens. The resulting, transformed A. tumefaciens can then be used to transform higher plants such as tobacco wherein the
10 transit peptide-linked enzyme is expressed and transporated to chloroplasts for the production of zeaxanthin.

b. Recombinant DNA Molecules

15 A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment defining a structural gene capable of expressing the Erwinia herbicola enzyme beta-carotene hydroxylase and a promoter suitable for driving the expression of that
20 enzyme in a compatible host organism, is also contemplated by this invention. The structural gene has a nucleotide base sequence described above. Particularly preferred recombinant DNA molecules include E. coli plasmid pARC404BH that contains the
25 about 874 bp Nco I-Sma I fragment, E. coli plasmid pARC406BH that includes that same restriction fragment driven by the Rec 7 promoter, and plasmid pARC145H designed for S. cerevisiae expression of GAL 1- or GAL 10-driven or PGK-driven and URA 3-terminated beta-carotene hydroxylase, as well as expression of GGPP
30 synthase and phytoene synthase driven by the GAL 1 and GAL 10 promoters.

7. Zeaxanthin Glycosylase Gene and Plasmid Construct

a. DNA Segment

Also contemplated by this invention is an isolated DNA segment comprising a nucleotide sequence that contains at least about 1200 base pairs, including a sequence defining a structural gene capable of expressing the Erwinia herbicola enzyme zeaxanthin glycosylase, an enzyme that synthesizes zeaxanthin diglucoside, and variant DNAs. The native DNA sequence for Erwinia herbicola lies between positions 10232 and 9033 of plasmid pARC376. A preferred DNA encoding zeaxanthin glycosylase is in the about 1390 bp Nde I-Ava I fragment of plasmid pARC2019. The DNA sequence of the E. herbicola structural gene is shown in Figure 25 (SEQ ID NO:97).

A further particularly preferred DNA segment is in an Xba I-Xba I fragment including about 2127 bp constituted by the following sequence of genes: (a) the about 450 bp CaMV 35S promoter, (b) the 177 bp sequence that encodes a chloroplast transit peptide operatively linked in frame to the 5' end of (c) the about 1200 bp zeaxanthin glycosylase gene, and (d) the about 300 bp NOS polyadenylation sequence. This Xba I fragment can be cloned into the Xba I site of plasmid pGA482, with the resulting plasmid being used to transform A. tumefaciens. The resulting, transformed A. tumefaciens can then be used to transform higher plants such as tobacco wherein the transit peptide-linked enzyme is expressed and transported to chloroplasts for the production of zeaxanthin diglucoside.

-35-

b. Recombinant DNA Molecules

A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment defining a structural gene capable of expressing the Erwinia
5 herbicola enzyme zeaxanthin glycosylase and a promoter suitable for driving the expression of that enzyme in a compatible host organism, is also contemplated by this invention. The structural gene has a nucleotide base sequence described above. Particularly preferred
10 recombinant DNA molecules include the E. coli plasmid pARC2019 that contains the about 1390 bp Nde I-Ava I fragment, an E. coli plasmid driven by the Rec 7 promoter, and a plasmid designed for S. cerevisiae expression of a GAL-1-, GAL-10- or PGK-driven
15 zeaxanthin glycosylase.

8. DNA Size

The previously described DNA segments are noted as having a minimal length, as well as total
20 overall lengths. That minimal length defines the length of a DNA segment having a sequence that encodes a particular protein enzyme. Inasmuch as the coding sequences for each of the six genes disclosed herein are illustrated in the accompanying figures, isolated
25 DNA segments and variants thereof can be prepared by in vitro mutagenesis, as described in the examples, that begin at the initial ATG codon for a gene and end at or just downstream of the stop codon for each gene. Thus, a desired restriction site can be engineered at or
30 upstream of the initiation codon, and at or downstream of the stop codon so that shorter structural genes than most of those discussed above can be prepared, excised and isolated.

As is well known in the art, so long as the
35 required DNA sequence is present, (including start and

stop signals), additional base pairs can be present at either end of the segment and that segment can still be utilized to express the protein. This, of course, presumes the absence in the segment of an operatively
5 linked DNA sequence that represses expression, expresses a further product that consumes an enzyme desired to be expressed, expresses a product that consumes a wanted reaction product produced by that desired enzyme, or otherwise interferes with the
10 structural gene of the DNA segment.

Thus, so long as the DNA segment is free of such interfering DNA sequences, a DNA segment of the invention can be 2,000-15,000 base pairs in length. The maximum size of a recombinant DNA molecule,
15 particularly an expression vector, is governed mostly by convenience and the vector size that can be accommodated by a host cell, once all of the minimal DNA sequences required for replication and expression, when desired, are present. Minimal vector sizes are
20 well known. Such long DNA segments are not preferred, but can be used.

Example 4b illustrates that a DNA segment of several thousand base pairs that contains the structural genes for GGPP synthase and phytoene
25 synthase can be used to produce phytoene. The same situation is true for phytoene dehydrogenase-4H production as is seen in Example 9b. The DNA segment used in Example 9b contains structural genes for GGPP synthase, phytoene synthase and phytoene dehydrogenase-
30 4H, lycopene cyclase and the other structural genes for zeaxanthin preparation. However, the gene for lycopene cyclase, which utilizes lycopene, was impaired so that no functional lycopene cyclase was produced and lycopene accumulated. A similar situation is
35 illustrated in Example 16b wherein the gene for

-37-

β -carotene hydroxylase originally present in plasmid pARC376 was made inoperative and β -carotene was found to accumulate.

5 **9. Construction of Plasmids**

b. DNA segments

 DNA segments that encode the before-described enzyme proteins can be synthesized by chemical techniques, for example, the phosphotriester method of
10 Matteucci et al., J. Am. Chem. Soc., 103:3185 (1981).
 (The disclosures of the art cited herein are incorporated herein by reference.) Of course, by chemically synthesizing the coding sequence, any
15 desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. However, DNA segments including sequences discussed previously are preferred.

 Furthermore, DNA segments containing
20 structural genes encoding the enzyme proteins can be obtained from recombinant DNA molecules (plasmid vectors) containing those genes. For instance, the plasmid type recombinant DNA molecules pARC417BH, pARC489B, pARC489D, pARC285, and pARC140N each contain
25 DNA sequences encoding different portions of the GGPP synthase and phytoene synthase proteins and together possess the entire sequence of DNA necessary for expression of either protein in biologically active form. Plasmid pARC145G contains DNA segments encoding
30 both enzymes. In addition, the plasmid type recombinant DNA molecules pARC496A, pARC146D, pATC228 and pATC1616 each contain a DNA sequence encoding biologically active phytoene dehydrogenase-4H proteins. Similarly, the plasmid type recombinant DNA molecules
35 pARC1509, pARC1510, and pARC1520 each contain a DNA

sequence encoding biologically active lycopene cyclase proteins. Also, the plasmid type recombinant DNA molecules pARC404BH, pARC406BH and pARC145H each contain a DNA sequence encoding biologically active
5 beta-carotene hydroxylase proteins, whereas plasmid pARC2019 contains a DNA sequence that encodes biologically active zeaxanthin glycosylase.

Plasmids pARC417BH, pARC489B, pARC489D, pARC285, pARC140N and pARC145G have been deposited
10 pursuant to Budapest Treaty requirements with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 on February 26, 1990 and were assigned the following respective accession numbers 40755, 40758, 40757, 40756, 40759, and 40753.
15 Plasmids pARC496A, pARC146D and pATC228 were deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection, (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 on May 11, 1990 and were assigned the following respective accession numbers
20 40803, 40801 and 40802. Plasmid pATC1616 was similarly deposited on May 15, 1990 and was assigned accession No. 40806. Also, plasmids pARC1509, pARC1510, and pARC1520 were deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection,
25 (AT) 12301 Parklawn Drive, Rockville, MD 20852 on July 27, 1990 and were assigned the following respective accession numbers 40850, 40851 and 40852. Plasmids pARC404BH, pARC406BH and pARC145H were similarly deposited on January 16, 1991, and were assigned ATCC
30 accession numbers 40943, 40945, and 40944, respectively. Plasmid pARC2019 was also deposited in accordance with the Budapest Treaty on February 13, 1991 and received ATCC accession number 40974.

A DNA segment that includes a DNA sequence
35 encoding zeaxanthin glycosylase, beta-carotene

hydroxylase, lycopene cyclase, phytoene dehydrogenase-4H, GGPP synthase, and phytoene synthase can be prepared by excising and operatively linking appropriate restriction fragments from each of the above deposited plasmids using well known methods. The DNA molecules of the present invention produced in this manner typically have cohesive termini, i.e., "overhanging" single-stranded portions that extend beyond the double-stranded portion of the molecule. The presence of cohesive termini on the DNA molecules of the present invention is preferred, although molecules having blunt termini are also contemplated.

Ribonucleic acid (RNA) equivalents of the above described DNA segments are also contemplated.

c. Recombinant DNA Molecules

A recombinant DNA molecule of the present invention can be produced by operatively linking a vector to a DNA segment of the present invention to form a plasmid such as those discussed and deposited herein. Particularly preferred recombinant DNA molecules are discussed in detail in the examples, hereafter. Vectors capable of directing the expression of GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and/or zeaxanthin glycosylase genes are referred to herein as "expression vectors".

The expression vectors described above contain expression control elements including the promoter. The polypeptide coding genes are operatively linked to the expression vector to allow the promoter sequence to direct RNA polymerase binding and expression of the desired polypeptide coding gene. Useful in expressing the polypeptide coding gene are promoters which are inducible, viral, synthetic, constitutive as described

by Poszkowski et al., EMBO J., 3:2719 (1989) and Odell et al., Nature, 313:810 (1985), and temporally regulated, spatially regulated, and spatiotemporally regulated as given in Chua et al., Science, 244:174-181 (1989).

The choice of which expression vector and ultimately to which promoter a polypeptide coding gene is operatively linked depends directly on the functional properties desired, e.g. the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the replication, and preferably also the expression (for an expression vector) of the polypeptide coding gene included in the DNA segment to which it is operatively linked.

In one preferred embodiment, a vector includes a prokaryotic replicon; i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell transformed therewith. Such replicons are well known in the art.

Those vectors that include a prokaryotic replicon can also include a prokaryotic promoter region capable of directing the expression of the GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase or zeaxanthin glycosylase genes in a host cell, such as E. coli, transformed therewith. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing one or more convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are

-41-

pUC8, pUC9, and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223-3 available from Pharmacia, Piscataway, N.J. A particularly preferred promoter for use in prokaryotic cells such as E. coli is the Rec 7 promoter present in plasmid vectors pARC306A, pARC496A and pARC136, and inducible by exogenously supplied nalidixic acid.

Expression vectors compatible with eukaryotic cells, preferably those compatible with yeast cells or more preferably those compatible with cells of higher plants, are also contemplated herein. Such expression vectors can also be used to form the recombinant DNA molecules of the present invention. Vectors for use in yeasts such as S. cerevisiae can be episomal or integrating, as is well known. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources.

Normally, such vectors contain one or more convenient restriction sites for insertion of the desired DNA segment and promoter sequences. Exemplary promoters for use in S. cerevisiae include the S. cerevisiae phosphoglyceric acid kinase (PGK) promoter and the divergent promoters GAL 10 and GAL 1.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of Agrobacterium tumefaciens described by Rogers et al., Meth. in Enzymol., 153:253-277 (1987). However, several other expression vector systems are known to function in plants including pCaMVN transfer control vector described by Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985). Plasmid pCaMVN (available from Pharmacia, Piscataway, NJ) includes the cauliflower mosaic virus CaMV 35S promoter. The

introduction of genes into higher plants is discussed in greater detail hereinafter.

5 The use of retroviral expression vectors to form the recombinant DNAs of the present invention is also contemplated. As used herein, the term "retroviral expression vector" refers to a DNA molecule that includes a promoter sequence derived from the long terminal repeat (LTR) region of a retrovirus genome.

10 Since some of these carotenoid products are to be associated with food production and coloration, the retroviral expression vector is preferably replication-incompetent in eukaryotic cells. The construction and use of retroviral vectors has been described by Verma, PCT Publication No. WO87/00551, and Cocking et al,
15 Science, 236:1259-62 (1987).

In preferred embodiments, the vector used to express the polypeptide coding gene includes a selection marker that is effective in a plant cell, preferably a drug resistance selection marker. One
20 preferred drug resistance marker is the gene whose expression results in kanamycin resistance, i.e., the chimeric gene containing the nopaline synthase promoter, Tn5 neomycin phosphotransferase II and nopaline synthase 3' nontranslated region described by
25 Rogers et al., in Methods For Plant Molecular Biology, A. Weissbach and H. Weissbach, eds., Academic Press Inc., San Diego, CA (1988). Another preferred marker is the assayable chloramphenicol acetyltransferase (cat) gene from the transposon Tn9.

30 A variety of methods has been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The
35 vector and DNA segment are then joined by hydrogen

-43-

bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Alternatively, synthetic linkers containing one or more restriction endonuclease sites can be used to join the DNA segment to the expression vector. The synthetic linkers are attached to blunt-ended DNA segments by incubating the blunt-ended DNA segments with a large excess of synthetic linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying synthetic linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction endonuclease and ligated into an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the synthetic linker. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including New England BioLabs, Beverly, MA.

Also contemplated by the present invention are RNA equivalents of the above described recombinant DNA molecules.

25 d. Introducing genes into higher plants

Methods for introducing polypeptide coding genes into higher, multicelled plants include Agrobacterium-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant species may not necessarily be the most effective for another plant

species, but it is well known which methods are useful for a particular plant species.

5 Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of Agrobacterium-mediated expression vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley et al., Biotechnology, 3:629 (1985) and Rogers et al., Methods in Enzymology, 153:253-277 (1987). Further, the integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome as described by Spielmann et al., Mol. Gen. Genet., 205:34 (1986) and Jorgensen et al., Mol. Gen. Genet., 207:471 (1987).

20 Modern Agrobacterium transformation vectors are capable of replication in E. coli as well as Agrobacterium, allowing for convenient manipulations as described by Klee et al., in Plant DNA Infectious Agents, T. Hohn and J. Schell, eds., Springer-Verlag, New York (1985) pp. 179-203.

25 Moreover, recent technological advances in vectors for Agrobacterium-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described by Rogers et al., Methods in Enzymology, 153:253 (1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted

polypeptide coding genes and are suitable for present purposes.

5 In those plant species where Agrobacterium-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer. However, few monocots appear to be natural hosts for Agrobacterium, although transgenic plants have been produced in asparagus using Agrobacterium vectors as described by Bytebier et al.,
10 Proc. Natl. Acad. Sci. U.S.A., 84:5345 (1987). Therefore, commercially important cereal grains such as rice, corn, and wheat must be transformed using alternative methods.

Higher plants have the ability to produce
15 carotenoids. The site of synthesis for all plant carotenoids is in the chloroplast. Carotenoid biosynthesis is highly regulated in plants. Masoner et al., Planta 105:267 (1972); Frosch et al., Planta 148:279 (1980); Mohr, Photosynthesis V. Chloroplast Development, pp. 869-883 (1981); Oelmueller et al.,
20 Planta 164:390 (1985); Harpster et al., Physiol. Plant. 64:147 (1985); Steinmueller et al., Molecular Form and Function of the Plant Genome, pp. 277-290 (1986). Therefore, the ability to use recombinant DNA
25 technology to increase endogenous carotenoid biosynthesis is questionable unless a novel approach is used. However, using the genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin
30 glycosylase to induce zeaxanthin and zeaxanthin diglucoside synthesis in the cytoplasm is a viable approach, even though carotenoids are not naturally produced in the cytoplasm.

Agrobacterium-mediated transformation of leaf
35 disks and other tissues appears to be limited to plant

species that Agrobacterium naturally infects. Thus, Agrobacterium-mediated transformation is most efficient in dicotyledonous plants. However, as mentioned above, the transformation of asparagus using Agrobacterium can also be achieved. See, for example, Bytebier, et al., Proc. Natl. Acad. Sci., 84:5345 (1987).

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments. See, for example, Potrykus et al., Mol. Gen. Genet., 199:183 (1985); Lorz et al., Mol. Gen. Genet., 199:178 (1985); Fromm et al., Nature, 319:791 (1986); Uchimiya et al., Mol. Gen. Genet., 204:204 (1986); Callis et al., Genes and Development, 1:1183 (1987); and Marcotte et al., Nature, 335:454 (1988).

Application of these systems to different plant species depends upon the ability to regenerate that particular plant species from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described in Fujimura et al., Plant Tissue Culture Letters, 2:74 (1985); Toriyama et al., Theor. Appl. Genet., 73:16 (1986); Yamada et al., Plant Cell Rep., 4:85 (1986); Abdullah et al., Biotechnology, 4:1087 (1986).

To transform plant species that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described by Vasil, Biotechnology, 6:397 (1988). In addition, "particle gun" or high-velocity microprojectile technology can be utilized. Using such technology, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal

particles as described in Klein et al., Nature, 327:70 (1987); Klein et al., Proc. Natl. Acad. Sci. U.S.A., 85:8502 (1988); and McCabe et al., Biotechnology, 6:923 (1988). The metal particles penetrate through several
5 layers of cells and thus allow the transformation of cells within tissue explants.

Metal particles have been used to successfully transform corn cells and to produce fertile, stably transformed tobacco and soybean plants. Transformation
10 of tissue explants eliminates the need for passage through a protoplast stage and thus speeds the production of transgenic plants.

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou et al., Methods in Enzymology, 101:433 (1983); D. Hess,
15 Intern Rev. Cytol., 107:367 (1987); Luo et al., Plant Mol. Biol. Reporter, 6:165 (1988). Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as
20 described by Pena et al., Nature, 325:274 (1987). DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus et al., Theor. Appl. Genet., 75:30 (1987); and Benbrook et al., in
25 Proceedings Bio Expo 1986, Butterworth, Stoneham, MA, pp. 27-54 (1986).

The regeneration of plants from either single plant protoplasts or various explants is well known in the art. See, for example, Methods for Plant Molecular
30 Biology, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, CA (1988). This regeneration and growth process includes the steps of selection of transformant cells and shoots, rooting the transformant shoots and growth of the plantlets in soil.

-48-

The regeneration of plants containing the foreign gene introduced by Agrobacterium from leaf explants can be achieved as described by Horsch et al., Science, 227:1229-1231 (1985). In this procedure, transformants are grown in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant species being transformed as described by Fraley et al., Proc. Natl. Acad. Sci. U.S.A., 80:4803 (1983).

This procedure typically produces shoots within two to four weeks and these transformant shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Transformant shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant species employed, such variations being well known in the art.

In higher plants, the transformed elements are so manipulated as to permit them to mature into soil- or otherwise-cultivated plants, such as plants that are cultivated hydroponically or in other soil-free media such as lava rock, crushed coral, sphagnum moss and the like.

Methods not utilizing tissue culture procedures are also contemplated, for example, using Agrobacterium-mediated vectors to produce transgenic plants from seeds.

A plant of the present invention containing one or more of the desired six enzyme proteins; i.e., GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, β -carotene hydroxylase and zeaxanthin glycosylase, is cultivated using methods well known to one skilled in the art.

Any of the transgenic plants of the present invention can be cultivated to isolate the desired carotenoid products they contain.

After cultivation, the transgenic plant is
5 harvested to recover the carotenoid product. This harvesting step can consist of harvesting the entire plant, or only the leaves, or roots of the plant. This step can either kill the plant or if only a non-essential portion of the transgenic plant is harvested
10 can permit the remainder of the plant to continue to grow.

In preferred embodiments this harvesting step further comprises the steps of:

(i) homogenizing at least a carotenoid-
15 containing portion of the transgenic plant to produce a plant pulp and using the carotenoid-containing pulp directly, as in dried pellets or tablets as where an animal food is contemplated; or

(ii) extracting the carotenoid(s) from the
20 plant pulp with an appropriate solvent such as an organic solvent or by supercritical extraction [Favati et al., J. Food Sci., 53:1532 (1988) and the citations therein] to produce a carotenoid-containing liquid solution or suspension; and

(iii) isolating the carotenoid(s) from the
25 solution or suspension.

At least a portion of the transgenic plant is homogenized to produce a plant pulp using methods well known to one skilled in the art. This homogenization
30 can be done manually, by a machine, or by a chemical means as long as the transgenic plant portions are broken up into small pieces to produce a plant pulp. This plant pulp consists of a mixture of the carotenoid of interest residual amounts of precursors, cellular
35 particles and cytosol contents. This pulp can be dried

and compressed into pellets or tablets and eaten or otherwise used to derive the benefits, or the pulp can be subjected to extraction procedures.

5 The carotenoid can be extracted from the
plant pulp produced above to form a solution or
suspension. Such extraction processes are common and
well known to one skilled in this art. For example,
the extracting step can consist of soaking or immersing
10 the plant pulp in a suitable solvent. This suitable
solvent is capable of dissolving or suspending the
carotenoid present in the plant pulp to produce a
carotenoid-containing solution or suspension. Solvents
useful for such an extraction process are well known to
those skilled in the art and include water, several
15 organic solvents and combinations thereof such as
methanol, ethanol, isopropanol, acetone, acetonitrile,
tetrahydrofuran (THF), hexane, and chloroform. A
vegetable oil such as peanut, corn, soybean and similar
oils can also be used for this extraction.

20 Isolation (harvesting) of carotenoids from
bacteria, yeasts, fungi and other lower organisms is
illustrated hereinafter using A. tumefaciens and
E. coli. Broadly, cells transfected with structural
genes for GGPP synthase, phytoene synthase, phytoene
25 dehydrogenase-4H, lycopene cyclase, beta-carotene
hydroxylase and zeaxanthin glycosylase, as desired, are
grown under suitable conditions for a period of time
sufficient for a desired carotenoid to be synthesized.
The carotenoid-containing cells, preferably in dried
30 form, are then lysed chemically or mechanically, and
the carotenoid is extracted from the lysed cells using
a liquid organic solvent, as described before, to form
a carotenoid-containing liquid solution or suspension.
The carotenoid is thereafter isolated from the liquid

-51-

solution or suspension by usual means such as chromatography.

The carotenoid is isolated from the solution or suspension produced above using methods that are well known to those skilled in the art of carotenoid isolation. These methods include, but are not limited to, purification procedures based on solubility in various liquid media, chromatographic techniques such as column chromatography and the like.

D. Methods for Preparing Carotenoid Biosynthesis

Enzymes

1. Introduction

a. Transformed Cells and Cultures

The present invention also relates to host cells transformed with recombinant DNA molecules of the present invention, preferably recombinant DNA capable of expressing GGPP synthase and membrane-bound (or soluble) phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase enzymes. These six enzymes can be referred to as carotenoid biosynthesis enzymes.

The host cells can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of E. coli such as, for example the E. coli strain HB101, available from BRL Life Technologies, Inc., Gaithersburg, MD (BRL). Preferred eukaryotic host cells include yeast and plant cells or protoplasts, preferably cells from higher plants. Preferred eukaryotic host cells include S. cerevisiae cells such as YPH499 obtained from Dr. Phillip Hieter, Johns Hopkins University, Baltimore, MD, discussed in Example 5.

Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is

accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen et al., Proc. Natl. Acad. Sci. USA, 69:2110 (1972); and Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of plant cells with retroviral vectors containing recombinant DNAs, see, for example, Verma, PCT Publication No. WO 87/00551, 1987, who isolated protoplasts from plant tissue, and inserted the retroviral genome in proviral (double stranded) form into the genome of the protoplasts. The transformed protoplasts were developed into callus tissue and then regenerated into transgenic plants. Plants derived from the protoplasts and their progeny carry the genetic material of the recombinant retroviral vector in their genomes and express the protein product.

Successfully transformed cells; i.e., cells that contain a recombinant DNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of a recombinant DNA of the present invention can be cloned to produce monoclonal colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the recombinant DNA using a method such as that described by Southern, J. Mol. Biol., 98:503 (1975) or Berent et al., Biotech., 3:208 (1985).

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of specific protein antigens. For example, cells successfully transformed with an expression

-53-

vector may produce proteins displaying GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase or zeaxanthin glycosylase antigenicity.

5 Identifying successful transformation of E. coli in this invention is relatively easy for carotenoids, except phytoene. Carotenoid-containing colonies formed are usually characterized by colored pigment formation. For example, beta-carotene,
10 zeaxanthin and zeaxanthin diglucoside are yellow and lycopene is red.

b. Methods for Producing Enzymes

A method is contemplated by this invention for
15 preparing a carotenoid biosynthesis enzyme. This method comprises initiating a culture, in a nutrient medium, of transformed prokaryotic or eukaryotic host cells. The host cells are transformed with a recombinant DNA molecule containing a compatible
20 expression vector operatively linked to a before-described exogenous DNA segment that defines the structural gene for a carotenoid biosynthesis enzyme, as desired.

This invention further comprises cultures
25 maintained for a time period sufficient for the host cells to express the carotenoid biosynthesis enzyme protein molecules, which proteins can be recovered in purified form if desired. Nutrient media useful for culturing transformed host cells are well known in the
30 art and can be obtained from several commercial sources. A further discussion of useful host cells and nutrient media are provided in the following section.

A further aspect contemplated is a method for
35 preparing one carotenoid biosynthesis enzyme in the

presence of either or all of the other carotenoid biosynthesis enzymes such as GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, β -carotene hydroxylase and zeaxanthin glycosylase.

5 This method is substantially identical to the before-described method except that the host cells are also transformed with a compatible expression vector operatively linked to a before-described exogenous DNA segment that defines any or all of the structural genes
10 for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, β -carotene hydroxylase and zeaxanthin glycosylase.

The transformed host cell can contain a single expression vector that contains one or more of the six
15 structural genes. The host can also be transformed with two expression vectors each containing a structural gene for one or more of the enzymes; e.g., one for at least beta-carotene hydroxylase or zeaxanthin glycosylase and another that contains at
20 least one of the other four (or five) enzymes, or three expression vectors; e.g., one for at least beta-carotene hydroxylase or zeaxanthin glycosylase, and two others that each contain at least one of the other four (or five) enzymes. An exemplary four expression vector
25 transformation can also be used in which at least one expression vector contains the structural gene that encodes beta-carotene hydroxylase or zeaxanthin glycosylase, with each of the other vectors containing at least one structural gene for each of the other
30 structural genes. A host cell can also be transformed with five vectors; i.e., one expression vector that contains the gene encoding each one of the first four enzymes and another vector containing the last two genes. A six-vector system can also be utilized for
35 production of zeaxanthin glycosylase in which a host is

transformed with one expression vector for each of the six named enzymes.

2. Methods for Preparing Carotenoids

5 A carotenoid can be produced by a method that includes initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells that are transformed with a recombinant DNA molecule containing a compatible expression vector operatively linked to a
10 before-described exogenous DNA segment that defines the structural gene for a carotenoid biosynthesis enzyme that converts an immediate precursor substrate molecule into the desired carotenoid, and which cells provide the immediate precursor molecule that is the substrate
15 for the expressed carotenoid biosynthesis enzyme. The cell culture is maintained for a time period sufficient for the transformed cells to produce (express) the desired carotenoid biosynthesis enzyme, and for that expressed enzyme to convert the provided immediate
20 precursor substrate molecule into the desired carotenoid. The produced carotenoid can thereafter be recovered as discussed herein. In higher plants, the nutrient medium (and in many cases the enzyme substrate that is the immediate precursor molecule) is supplied
25 by the plant itself, and the initiated culture is the germinated seed, protoplast or even a grafted explant from a prior culture.

 In one embodiment where the host cells do not themselves produce carotenoid biosynthesis enzymes or
30 immediate precursor substrate molecules, the required carotenoid biosynthesis enzymes are provided to the cells by transformation of those cells with one or more exogenous recombinant DNA molecules that encode and express the appropriate genes so that appropriate
35 enzymes and precursor substrate molecules are provided

-56-

to the cells. For example, in E. coli, an exogenous structural gene for GGPP synthase is required to produce GGPP from the ubiquitous precursor FPP, and an exogenous structural gene for phytoene synthase is required to convert GGPP (the immediate precursor substrate) into phytoene. Thus, at least two exogenous recombinant DNA molecules are needed to produce a carotenoid.

If the next carotenoid shown in Figure 1, lycopene, is desired, the above transformed cells must also be further transformed with an exogenous recombinant DNA molecule that codes for and expresses phytoene dehydrogenase-4H. Further transformation of such transformed cells is needed as each ensuing carotenoid illustrated in Figure 1 that is desired to be prepared. Thus, with transformation with the six structural genes discussed herein is accomplished for the production of zeaxanthin diglucoside. The exogenous structural genes used for the transformation can reside in a single recombinant DNA molecule, or in a plurality of such recombinant molecules as is exemplified below.

In one aspect for producing phytoene, the recombinant DNA molecule contains an expression system that comprises one or more expression vectors compatible with host cells operatively linked to an exogenous DNA segment that comprises (i) a nucleotide base sequence corresponding to a sequence defining a structural gene for GGPP synthase as discussed before, and (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase as also discussed before. A particularly preferred expression vector plasmid pARC145G contains structural genes for both GGPP synthase and phytoene synthase, and produces phytoene in S. cerevisiae.

In another aspect for producing lycopene, the recombinant DNA molecule preferably contains an expression system that comprises one or more expression vectors compatible with host cells, operatively linked to an exogenous DNA segment that comprises (i) a nucleotide base sequence corresponding to a sequence, defining a structural gene for GGPP synthase, and (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase, and (iii) a nucleotide base sequence corresponding to the sequence defining a structural gene for phytoene dehydrogenase-4H. Thus, phytoene is provided to the host cells by the enzymes expressed by the expression system.

In one particularly preferred aspect, the structural genes for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H are contained operatively linked in a single expression vector, preferably under the control of the same promoter. In another preferred aspect, two expression vectors are used, with the structural genes for GGPP synthase and phytoene synthase on one vector and the structural gene for phytoene dehydrogenase-4H on the other vector. In yet another preferred embodiment, three expression vectors are used. Yeast and plants require a separate promoter for each gene, although the same promoter can be used for each gene.

Example 9b illustrates lycopene production in E. coli host cells using a single expression vector (pARC376-Ava 102) containing all three genes. Similarly, the very active GGPP synthase gene contained in plasmid pARC489D and phytoene synthase gene contained in plasmid pARC140N can be transformed separately or together with the phytoene dehydrogenase-4H structural gene found in plasmid pARC496A to prepare

-58-

transformed host E. coli cells that contain all three functional structural genes. Here, expression of plasmids pARC489D and pARC140N provides the enzymes needed to convert ubiquitous cellular precursors into the required phytoene that is converted into lycopene by the action of the phytoene dehydrogenase-4H expressed by plasmid pARC496A. Likewise, Example 10 illustrates lycopene production in S. cerevisiae host cells transformed with both plasmid pARC145G, whose expression products provides phytoene to the cells, and plasmid pARC146D that expresses phytoene dehydrogenase-4H that converts the provided phytoene into lycopene.

In yet another aspect for the production of β -carotene, the recombinant DNA molecule preferably contains an expression system that comprises one or more expression vectors compatible with host cells, operatively linked to an exogenous DNA segment that comprises (i) a nucleotide base sequence corresponding to a sequence, defining a structural gene for GGPP synthase, and (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase, (iii) a nucleotide base sequence corresponding to the sequence defining a structural gene for phytoene dehydrogenase-4H, and (iv) a nucleotide base sequence corresponding to the sequence defining a structural gene for lycopene cyclase. Thus, lycopene is provided to the host cells by the enzymes expressed by the expression system.

In one particularly preferred aspect, the structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase are contained operatively linked in a single expression vector, preferably under the control of the same promoter. In another preferred aspect, two expression vectors are used, with the structural genes for GGPP

synthase, phytoene synthase and phytoene dehydrogenase-4H on one vector and the structural gene for lycopene cyclase on the other vector. In yet another preferred aspect, three expression vectors are used. Yeast and
5 plants require a separate promoter for each gene, although the same promoter can be used for each gene.

Example 16 illustrates beta-carotene production in E. coli host cells using a single expression vector plasmid pARC376-Pst 102 containing
10 all four genes. Similarly, the very active GGPP synthase gene contained in plasmid pARC489D, phytoene synthase gene contained in plasmid pARC140N and the phytoene dehydrogenase-4H structural gene found in plasmid pARC496A can be transformed separately or
15 together with the lycopene cyclase structural gene found in plasmid pARC1510 to prepare transformed host E. coli cells that contain all four functional structural genes. Here, expression of plasmids pARC489D, pARC140N and pARC496A provides the enzymes
20 needed to convert ubiquitous cellular precursors into the required phytoene that is converted into lycopene that is subsequently converted into beta-carotene by the action of the lycopene cyclase expressed by plasmid pARC1510. Likewise, Example 17 illustrates beta-
25 carotene production in plasmid pARC145G, whose expression products provides phytoene to the cells and plasmid pARC1520 that expresses both phytoene dehydrogenase-4H, which converts the provided phytoene into lycopene, and lycopene cyclase that converts
30 lycopene into beta-carotene.

In a still further aspect, the recombinant DNA molecule preferably contains an expression system that comprises one or more expression vectors compatible with host cells, operatively linked to an exogenous DNA
35 segment that comprises (i) a nucleotide base sequence

corresponding to a sequence, defining a structural gene for GGPP synthase, and (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase, (iii) a nucleotide base sequence
5 corresponding to the sequence defining a structural gene for phytoene dehydrogenase-4H, (iv) a nucleotide base sequence corresponding to the sequence defining a structural gene for lycopene cyclase and (v) a
10 nucleotide base sequence corresponding to the sequence defining a structural gene for beta-carotene hydroxylase. Thus, beta-carotene is provided to the host cells by the enzymes expressed by the expression system.

In one particularly preferred aspect, the
15 structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase are contained operatively linked in a single expression vector, preferably under the control of the same promoter. In another preferred
20 aspect, two expression vectors are used, with the structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase on one vector and the structural gene for beta-carotene hydroxylase on the other vector. In yet another
25 preferred aspect, three expression vectors are used. Yeast and plants require a separate promoter for each gene, although the same promoter can be used for each gene.

Example 1 illustrates zeaxanthin production in
30 E. coli and A. tumefaciens host cells using a single expression plasmid vector pARC288 containing all five genes. Example 21 illustrates zeaxanthin production in
E. coli host cells using an expression plasmid vector pARC279 containing all four genes required to produce
35 beta-carotene, but with the gene for beta-carotene

-61-

hydroxylase having been deleted. E. coli containing plasmid pARC279 were further transformed with the plasmid pARC406BH, and then the cells were grown in appropriate selective medium. Zeaxanthin was produced.

5 Similarly, the very active GGPP synthase gene contained in plasmid pARC489D, the phytoene synthase gene contained in plasmid pARC140N, the phytoene dehydrogenase-4H gene found in plasmid pARC496A, the lycopene cyclase structural gene found in plasmid
10 pARC1510 can be transformed separately or together with the beta-carotene hydroxylase structural gene found in plasmid pARC406BH to prepare transformed host E. coli cells that contain all five functional structural genes. Here, expression of plasmids pARC489D,
15 pARC140N, pARC496A and pARC1510 provides the enzymes needed to convert ubiquitous cellular precursors into the required phytoene that is converted into lycopene and then beta-carotene, which is subsequently converted into zeaxanthin by the action of the beta-carotene
20 hydroxylase expressed by plasmid pARC406BH. Likewise, Example 22 illustrates zeaxanthin production in S. cerevisiae host cells transformed with plasmid pARC145H, whose expression products provide GGPP synthase, phytoene synthase and beta-carotene
25 hydroxylase to the cells, and plasmid pARC1520 that expresses both phytoene dehydrogenase-4H and lycopene cyclase. Thus all enzymes required for zeaxanthin biosynthesis are found on these two plasmids.

 In yet another aspect for preparing zeaxanthin
30 diglucoside, the recombinant DNA molecule preferably contains an expression system that comprises one or more expression vectors compatible with host cells, operatively linked to an exogenous DNA segment, comprising (i) a nucleotide base sequence corresponding
35 to a sequence, defining a structural gene for GGPP

synthase, and (ii) a nucleotide base sequence corresponding to a sequence defining a structural gene for phytoene synthase, (iii) a nucleotide base sequence corresponding to the sequence defining a structural gene for phytoene dehydrogenase-4H, (iv) a nucleotide base sequence corresponding to the sequence defining a structural gene for lycopene cyclase, (v) a nucleotide base sequence corresponding to a sequence defining a structural gene for beta-carotene hydroxylase, and a nucleotide base sequence corresponding to a sequence defining a structural gene for zeaxanthin glycosylase. Thus, zeaxanthin is provided to the host cells by the enzymes expressed by the expression system.

In one particularly preferred aspect, the structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase are contained operatively linked in a single expression vector, preferably under the control of the same promoter. In another preferred aspect, two expression vectors are used, with the structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase on one vector and the structural gene for zeaxanthin glycosylase on the other vector. In yet other preferred aspects, three, four, five or six expression vectors are used. Yeast and plants require a separate promoter for each gene, although the same promoter can be used for each gene.

Example 1 illustrates zeaxanthin diglucoside production in and recovery from E. coli using a single expression vector pARC376 containing all six genes. Example 26 illustrates zeaxanthin diglucoside production in E. coli host cells using expression vector pARC288 containing all five genes required to

-63-

produce zeaxanthin plus plasmid pARC2019 with the gene for zeaxanthin glycosylase. Thus, E. coli containing pARC288 were further transformed with the plasmid pARC2019, and then the cells were grown in appropriate selective medium. Zeaxanthin diglucoside was produced and recovered.

Similarly, the very active GGPP synthase gene contained in plasmid pARC489D, the phytoene synthase gene contained in plasmid pARC140N, the phytoene dehydrogenase-4H gene found in plasmid pARC496A, lycopene cyclase structural gene found in plasmid pARC1510 and the beta-carotene hydroxylase structural gene found in plasmid pARC406GH can be transformed separately or together with the zeaxanthin glycosylase gene found in plasmid pARC2019 to prepare transformed host E. coli cells that contain all six functional structural genes. Here, expression of plasmids pARC489D, pARC140N, pARC496A, pARC1510 and pARC406BH provides the enzymes needed to convert ubiquitous cellular precursors into the required phytoene that is converted into lycopene and then beta-carotene, that is converted into zeaxanthin and then into zeaxanthin diglucoside by the action of the zeaxanthin glycosylase expressed by plasmid pARC406BH. Likewise, Example 27 illustrates zeaxanthin diglucoside production in S. cerevisiae host cells multiply transformed with plasmid pARC145H, whose expression products provide GGPP synthase, phytoene synthase and beta-carotene hydroxylase to the cells, plasmid pARC1520 that expresses both phytoene dehydrogenase-4H and lycopene cyclase, and a plasmid that expresses zeaxanthin glycosylase. Thus all enzymes required for zeaxanthin diglucoside biosynthesis are found on these three plasmids.

The order of expression of the structural genes is not important so, for example, the structural gene for GGPP synthase can be located 5' (upstream) from the structural gene for phytoene synthase, or vice versa.

As another embodiment, this method also contemplates carotenoid production by use of transformed host cells containing only one carotenoid synthesis gene-containing expression vector. Here, the nutrient medium supplies the immediate precursor substrate molecule to the host cells so that those host cells can provide the precursor substrate for the expressed enzyme. The nutrient medium can contain the requisite amount of precursor in micelles or vesicles, as are well known, which are taken up by the host cells.

Another aspect of this embodiment contemplates host cells transformed with one, two, three, four or five expression vectors for the production of phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase. Here, GGPP is provided to the transformed host cells via the nutrient medium as above, and the transformed host cells convert the GGPP to the necessary phytoene and then to lycopene, beta-carotene, zeaxanthin and zeaxanthin diglucoside using the transformed structural genes. Of course, cells are transformed with fewer than all of the five genes where a carotenoid other than zeaxanthin diglucoside is desired and GGPP is provided by the medium to the cells.

It is understood in any of the methods of carotenoid production contemplated herein that the transformed host is free of exogenously supplied DNA segments that inhibit the production and/or accumulation of a desired carotenoid. Thus, for

-65-

example, where zeaxanthin is to be prepared,
exogenously supplied DNA that encodes a biologically
active zeaxanthin glycosylase that converts zeaxanthin
to zeaxanthin diglucoside is absent so that zeaxanthin
accumulates.

E. Examples

The following examples are intended to
illustrate, but not limit, the scope of the invention.
Studies related to carotenoid biosynthesis generally,
GGPP synthase and phytoene synthase are discussed in
Examples 1-7, studies related to lycopene are discussed
in Examples 8-14, studies related to beta-carotene are
discussed in Examples 15-20, studies related to
zeaxanthin are discussed in Examples 21-25, whereas
Examples 26-30 discuss zeaxanthin diglucoside.

All recombinant DNA techniques were performed
according to standard protocols as described in
Maniatis et al., Molecular Cloning, A Laboratory
Manual, Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY (1982), except where noted. All restriction
enzymes and other enzymes were used according to the
supplier's instructions. DNA sequencing was performed
on M13 single-stranded DNA using a modification of the
basic dideoxy method of Sanger et al, Proc. Natl. Acad.
Sci. U.S.A. 74:5463-7 (1977). A sequencing kit from
BRL Life Technologies, Inc., Gaithersburg, MD was used.
The DNA sequence was analyzed on the IG Suite from
Intelligenetics Corp.

Enzyme assays for enzymes engineered in
E. coli or Saccharomyces cerevisiae were performed
according to the protocols provided in Example 2e for
GGPP synthase and phytoene synthase, in Example 8g for
phytoene dehydrogenase-4H, and in Example 15f for
lycopene cyclase.

-66-

Carotenoids were extracted and analyzed by high performance liquid chromatography (HPLC) from both E. coli or S. cerevisiae according to the protocol provided in Example 4. The identity of zeaxanthin diglucoside was confirmed by mass spectroscopy performed according to the protocol provided in Example 4. The identity of zeaxanthin was confirmed by mass spectroscopy. The identification of the other carotenoids was confirmed by elution from HPLC, UV-Visible spectral analysis, and comparison with known standards of phytoene, lycopene, and beta-carotene.

The method for production in E. coli of the proteins in E. coli encoded by the different genes, using the inducible Rec 7 promoter system in the plasmid pARC306A, is described in Example 2d. These proteins were used in the enzyme assays described. This protocol was also used to produce sufficient amounts of the proteins from which the N-terminus of the protein was determined.

Examples 21-25 discuss the production of zeaxanthin. Example 21 describes construction of an engineered, readily movable structural gene for beta-carotene hydroxylase, whereas Example 22 illustrates the incorporation of that structural gene into plasmid pARC306A to form plasmid pARC404BH, which when placed into E. coli cells along with plasmid pARC279 caused production of zeaxanthin.

Examples 26-30 discuss the production of zeaxanthin diglucoside. Example 26 describes construction of an engineered, readily movable structural gene for zeaxanthin glycosylase, which when placed into E. coli along with plasmid pARC288 caused production of zeaxanthin diglucoside.

-67-

Example 1. Confirmation of the presence of the
carotenoid biosynthesis pathway genes in
Erwinia herbicola plasmid pARC376

a. E. coli

5 E. coli cells, which by themselves are not
capable of pigment formation, become intensely yellow
in color when transformed with plasmid pARC376 (Figure
5). The pigments responsible for the observed yellow
color were extracted from the cells and shown to be
10 zeaxanthin and zeaxanthin diglucosides from UV-VIS
spectral and mass spectral data.

 In the presence of diphenylamine in the growth
medium, pigment formation is strongly inhibited
resulting in colorless cells, which have been found to
15 accumulate trace amounts of phytoene. Diphenylamine is
known to inhibit the phytoene dehydrogenase-4H
reaction. This was the first indication that the
carotenoid pathway is functional in these transformed
cells. Harvesting mid-log phase cells and extracting
20 carotenoids from those cells indicated the presence of
phytoene, phytofluene, and zeta-carotene, further
confirming the presence of functional carotenoid
pathway syntheses in the cells.

25 **b. A. tumefaciens**

 Carotenoid production in A. tumefaciens
containing the Erwinia herbicola carotenoid DNA was
investigated. Three plasmids containing various
portions of plasmid pARC376 were transformed into A.
30 tumefaciens strain LBA4404. Four different carotenoids
were produced, i.e., phytoene, lycopene, beta-carotene,
and zeaxanthin.

 The three plasmids used in this study were:

1. Plasmid pARC803 (about 17 kb), which contained the
35 R1162 ori, the kanamycin resistance gene (NPTII)

-68-

- and the Erwinia herbicola DNA of plasmid pARC376-Ava 103 fragment (derived by deleting 2 Ava I restriction fragments, at about 8331-8842-10453, and cloning the Hind III (about 13463) to Eco RI (about 3370 Figure 5) fragment into plasmid pSOC925 (Figure 12);
- 5
2. Plasmid pARC274 (about 17 kb), which contained the R1162 ori, the kanamycin resistance gene, and the Erwinia herbicola DNA of plasmid pARC376-Bam 100 fragment (derived by deleting 2 Bam HI restriction fragments, at about 3442-4487-5302 and cloning the Hind III (about 13463) to Eco RI (about 3370, Figure 5) fragment into plasmid pSOC925;
- 10
3. Plasmid pARC288 (about 18 kb) which contained the R1162 ori, the kanamycin resistance gene, the Erwinia herbicola DNA of plasmid pARC376-Sal 8 (Example 2a) and the GGPP synthase gene fragment from Hind III (about 13463) to Eco RV (about 11196, Figure 5).
- 15
- 20

These plasmids were transformed into competent cells of Agrobacterium according to the protocol below.

- 25
1. An Agrobacterium colony was grown overnight (about 15 hours) in 2 to 3 ml YP medium (10 g/l Bactopeptone, 10 g/l yeast extracts, and 5 g/l NaCl, pH 7).
- 30
2. The overnight culture was transferred into 50 ml fresh YP medium in 250 ml flask at 250 rpm and 28°C, and grown until the culture reached 0.5 to 1.0 OD (A_{600}).
3. The culture was chilled on ice for 5 minutes, then the cells were harvested by centrifugation.

4. The cells were resuspended in 1 ml of 20 mM calcium chloride.
5. About 1 μ g of plasmid DNA was added into 0.1 ml of the cell suspension and mixture was incubated on ice for 30 minutes.
6. The reaction mixture was frozen in liquid nitrogen for 1 to 2 minutes and then incubated at 37°C for 5 minutes.
7. One ml of YP medium was added and the mixture was incubated at 28°C for 2 to 4 hours.
8. The cells were plated in LB medium (5 g/l yeast extracts, 10 g/l tryptone, 5 g/l NaCl, and 2 g/l glucose, pH 7) containing 50 μ g/ml kanamycin.

The transformed cells were selected on LB plates containing 50 μ g/ml of kanamycin at 28°C (LB plates = 10 g/l tryptone, 5 g/l yeast extracts, 5 g/l NaCl, 2 g/l glucose, and 15 g/l Bactoagar). The transformed cells were cultivated on the same rich medium for two days, harvested and dried for carotenoid extraction. For carotenoid extraction, 0.5 ml of water, 2.5 ml of acetone, and 2.5 ml of methanol were added to the dried cells. After 1 hour incubation with mixing at room temperature, the solvent containing carotenoids was filtered, and carotenoids isolated were analyzed by HPLC.

The carotenoids produced by both E. coli and Agrobacterium are listed in Table 1. The amounts of carotenoids produced by Agrobacterium were about 5 to 10 times lower than by E. coli cells carrying the same plasmids (by gross estimation).

-70-

Table 1
Carotenoids Produced by A. tumefaciens LBA4404

5	Plasmids	Major Carotenoids	
		E. coli	Agrobacterium
10	pARC803	Lycopene	Lycopene, Phytoene
	pARC274	β -Carotene	β -Carotene, (Phytoene)*
15	pARC288	Zeaxanthin	Zeaxanthin

* Minor component.

20 The origin of replication from plasmid R1162, described by Meyer, R. et al., J. Bacteriol. 152:140 (1982), was introduced into plasmid pARC376, to construct a broad host-range plasmid capable of replication in other bacteria. The resulting plasmid was used to introduce Erwinia herbicola carotenoid DNA into Rhodobacter sphaeroides and its carotenoid mutants. The results demonstrated that the Erwinia herbicola carotenoid DNA was not expressed in Rhodobacter cells, presumably because there was no complementation of the Rhodobacter phytoene synthase, phytoene dehydrogenase-4H and neurosporene dehydrogenase mutants. A further study, described hereinafter, indicated that phytoene dehydrogenase-4H could be expressed in Rhodobacter cells as hosts.

Example 2. GGPP Synthase Gene

The GGPP synthase gene was obtained from the pARC376 plasmid utilizing the following methods.

-71-

a. Digestion of pARC376 with Sal I

The plasmid pARC376-Sal 8 is a derivative of plasmid pARC376 from which two Sal I fragments were removed. One of those fragments is the approximately 1092 bp fragment bounded by the Sal I restriction sites at about 9340 and about 10432 shown in Figure 5, whereas the other is the 3831 bp (approximate size) fragment bounded by the Sal I restriction sites at about 10432 and about 14263 also in Figure 5. This was accomplished as follows.

Plasmid pARC376 DNA was prepared using the alkaline lysis method. 5 Micrograms of plasmid DNA were digested with Sal I (BRL) in a high salt buffer provided by the supplier and additionally containing 150 mM NaCl, for 1 hour at 37°C and purified on a 0.8 percent agarose gel. The remaining plasmid, about 10.2 kilobases in length, was electroeluted from the gel, phenol extracted and ethanol precipitated. After elimination of the above Sal I fragments from about positions 9340 to 14263, the remaining DNA was religated to itself to form plasmid pARC376-Sal 8.

b. Construction of pARC808

To determine if the gene for GGPP synthase was present on the deleted Erwinia herbicola DNA, plasmid pARC376-Sal 8 was cloned into plasmid pSOC925, an E. coli plasmid R1162 derivative, to generate plasmid pARC808. The plasmid pSOC925 contains the origin of replication from the R1162 plasmid, the NPT II gene from Tn5 that confers resistance to kanamycin, and unique Hind III and Eco RI restriction sites.

Briefly, the plasmid pSOC925 expression DNA vector was prepared for cloning by admixing 5 µg of plasmid DNA to a solution containing 5 units of each of the restriction endonucleases Hind III and Eco RI and

-72-

the Medium Salt Buffer from Maniatis. This solution was maintained at 37°C for 2 hours. The solution was heated at 65°C to inactivate the restriction endonucleases. The DNA was purified by extracting the solution with a mixture of phenol and chloroform followed by ethanol precipitation.

Plasmid pARC376-Sal 8 was digested with Hind III and Eco RI in a similar way. The Erwinia herbicola DNA in plasmid pARC376-Sal 8 from the Hind III site at about position 348 to the Eco RI site at about position 3370 (Figure 5) was then ligated into the plasmid vector pSOC925 that had already been digested with Hind III and Eco RI.

The ligation reaction contained about 0.1 µg of the plasmid vector pSOC925 and about 0.2 µg of the Erwinia herbicola Hind III to Eco RI fragment from plasmid pARC376-Sal 8 in a volume of 18 µl. Two µl of 10 X ligation buffer (IBI, Corp) and 2 units of T4 ligase were added. The ligation reaction was incubated at 4°C overnight (about 15 hours). The ligated DNA was transformed into E. coli HB 101 according to standard procedures (Maniatis). This generated the plasmid pARC808, which also codes for kanamycin resistance. The excised DNA fragment from plasmid pARC376-Sal 8 contains an endogenous promoter sequence upstream from the GGPP synthase gene.

Positive clones with inserts were identified by growing prospective positive clones, isolating plasmid DNA by the alkali lysis method (Maniatis), and performing restriction enzyme analysis on the isolated plasmid DNA's. E. coli cells transformed with this plasmid DNA did not produce colored carotenoids, as determined by visual inspection and HPLC and TLC analysis. Other studies discussed hereinafter demonstrated that plasmid pARC808 expresses Erwinia

-73-

herbicola enzymes that can convert phytoene into colored carotenoid pigments.

c. Construction of pARC282

5 A second plasmid was constructed by inserting
a restriction fragment containing the approximately
1153 bp Bgl II (about position 12349, Figure 5) to Eco
RV (about position 11196, Figure 5) fragment from
10 plasmid pARC376 into the Bam HI and Hind III sites of
pBR322 to produce plasmid pARC282. Briefly, the
plasmid pARC273 contains the Erwinia herbicola DNA from
the Bgl II site (at about position 12349) to the Eco RV
site (at about position 11196).

15 About 100 non-coding bp downstream from the
Eco RV site in plasmid pARC273 is a Hind III
restriction site, which is a part of the pARC273
vector. Here, about 5 µg of the plasmid pARC273 were
incubated with 5 units of each of the restriction
enzymes Bgl II and Hind III in the Medium Salt Buffer
20 (Maniatis) for 2 hours at 37°C. Five µg of the vector
pBR322 were incubated with 5 units of each of the
restriction enzymes Bam HI and Hind III in the Medium
Salt Buffer (Maniatis) for 2 hours at 37°C.

25 The Erwinia herbicola Bgl II to Hind III DNA
fragment (about 0.2 mg) from plasmid pARC273 was
admixed with the Bam HI and Hind III digested plasmid
pBR322 vector (about 0.1 µg) in 18 µl total volume.
Two µl of 10 X Ligation Buffer (IBI, Corp.) and 2 units
of T₄ Ligase were added, the reaction was incubated
30 overnight (about 15 hours) at 4°C, and the ligated DNA
was transformed into competent E. coli HB101 cells
according to procedures in Maniatis. Positive clones
were identified by growing the prospective
transformants, isolating plasmid DNA by the alkali

-74-

lysis method (Maniatis), and performing restriction enzyme analysis on the plasmid DNA.

This plasmid, pARC282 encodes ampicillin resistance in E. coli and includes a native Erwinia herbicola promoter between the Bgl II site and the initial Met codon of the GGPP synthase gene, but does not cause any carotenoids to be produced. However, when this plasmid was transferred into E. coli cells containing the plasmid pARC808, and the E. coli cells were grown in the presence of both kanamycin and ampicillin, carotenoids were synthesized as evidenced by production of the yellow pigment zeaxanthin. Thus, plasmid pARC282 contained the essential gene that was deleted from the plasmid pARC376-Sal 8 plasmid, and the presence of this gene in combination with other Erwinia herbicola carotenoid genes could restore carotenoid production in E. coli.

d. Other Plasmid Constructs

Enzyme assays were performed on similar plasmid constructs, including plasmid pARC491 which was constructed by cloning the approximately 1068 bp fragment from Hpa I (at about position 12264 of plasmid pARC376 or about position 84 of Figure 2) to Eco RV (at about position 11196, Figure 5) into a plasmid denominated pARC306A. Plasmid pARC306A, whose restriction map is illustrated in Figure 6 contains approximately 2519 base pairs. This plasmid contains the polylinker region from pUC18, a unique Nco I site, the ampicillin selectable marker, the pMB1 origin of replication and the Rec 7 promoter. Cells containing this plasmid construct had a level of 7.91 nmol/min/mg protein activity of GGPP synthase.

35

-75-

e. DNA sequencing

The accuracy of some of the cloning steps was confirmed by sequencing the insert using the dideoxy method described by Sanger et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467, (1977) and following the manufacturer's instructions included in a sequencing kit from BRL.

The DNA sequence was determined for the approximately 1153 base pair restriction fragment from the region between the Bgl II site at about 12349 of Figure 5 and the Eco RV site at about 11196 of Figure 5. The obtained DNA sequence and putative partial amino acid residue sequences are shown in Figure 2 (about positions 1 to 1153). The direction of transcription of the gene for GGPP synthase in plasmid pARC376 (Figure 5) is counterclockwise and proceeds in the direction from the Bgl II site toward the Eco RV site.

f. In Vitro mutagenesis

The initiation codon for GGPP synthase begins at about nucleotide position 12226 of plasmid pARC376 with the ATG codon for methionine (about position 124 of Figure 2). A Nco I restriction site was introduced at this position of the GGPP synthase gene using in vitro mutagenesis following the techniques described in Current Protocols In Molecular Biology, Ausabel et al. eds., John Wiley & Sons, New York, (1987) p. 8.1.1-8.1.6, with the exception that E. coli CJ 236 was grown (in step 3 at page 8.1.1) in further presence of 20 µg/µl chloramphenicol. The primer used was:

5' TCA GCG GGT AAC CTT GCC ATG GGG AGT GGC AGT AAA GCG 3'
Nco I site (SEQ ID NO:18)

-76-

The mutations were confirmed either by DNA sequencing or by the presence of the newly introduced Nco I site. This manipulation changed the natural sequence

5 TTG CAATGG TGA (SEQ ID NO:19) to
 TTG CCATGG GGA, (SEQ ID NO:20)

wherein a bold-faced letter above and in the following examples indicates an altered base.

10 This modified version of the GGPP synthase
gene from the newly introduced Nco I site to the Eco RV
site (about 1029 bp) was then inserted into the plasmid
pARC306A to generate plasmid pARC417BH. This plasmid,
pARC417BH, contains the E. coli promoter Rec 7 adjacent
to a multiple cloning site. Structural genes lacking a
15 promoter region, when introduced adjacent to the Rec 7
promoter, are expressed in E. coli.

When plasmid pARC417BH was introduced into E. coli
coli cells, GGPP synthase enzyme activity (measured as
GGOH) was found at the level of 6.35 nmol/min/mg
20 protein. In addition, when plasmid pARC417BH was
introduced into E. coli cells containing plasmid
pARC808, carotenoids were produced. This demonstrated
that the gene for GGPP synthase had been identified and
genetically engineered.

25

g. Fine tuning the GGPP synthase gene

Several constructs designed to express the
GGPP synthase gene were made to optimize the expression
of an active GGPP synthase enzyme. Again using in
30 vitro mutagenesis according to methods previously
cited, a Nco I site was introduced at about position
12264 of plasmid pARC376, using the primer,

5' CAT GGC GAA ATA GAA GCC ATG GGA CAA TCC ATT GAC GAT 3'
35 Nco I site (SEQ ID NO:21)

-77-

17 amino acids downstream from the initiation codon for the GGPP synthase gene that is located at about position 124 in Figure 2. That site was thus placed at the upstream side of the MET whose ATG codon begins at about position 175 of the sequence of Figure 2. The natural DNA sequence

AAG TAATGA GAC (SEQ ID NO:22) was changed to
AAG CCATGG GAC. (SEQ ID NO:23)

This modified GGPP synthase gene coding for seventeen fewer amino-terminal amino acid residues was inserted into plasmid pARC306A at the Nco I site of that plasmid to generate plasmid pARC418BH.

When GGPP synthase assays were performed on cells transformed with plasmid pARC418BH, no enzyme activity was detected. In addition, when this modified GGPP synthase was added to E. coli cells containing the plasmid having the rest of the genes for the enzymes required for carotenoid synthesis, plasmid pARC808 described above, no carotenoids were synthesized. This demonstrated that deletion of the 17 N-terminal amino acids of the GGPP synthase resulted in a non-functional enzyme.

A final construction was made at the 5' end of the GGPP synthase gene. A fragment excised from the GGPP synthase gene from the Nru I site (about 12187 of plasmid pARC376) through the Eco RV site (about 11196 of plasmid pARC376 or at about positions 162 through 1153 in Figure 2) to the Hind III site of plasmid pARC282 was inserted into the pARC306A plasmid, to form plasmid pARC489B that is discussed below.

Plasmid pARC306A was digested with Eco RI. The Eco RI end was converted to a blunt end using the Klenow fragment of DNA Pol I according to the usual techniques described by Maniatis. The Nru I blunt 5' end from the partially Nru I-Hind III fragment of

-78-

5 plasmid pARC282 was blunt end-ligated to the newly generated blunt end at the Eco RI site of plasmid pARC306A by mixing about 0.2 μ g of the Nru I to Eco RV Erwinia herbicola DNA with the pARC306A vector in about 18 μ l volume. Two μ l of 10X Ligation Buffer (IBI, Corp.) and two units of T4 Ligase were added, and the reaction was incubated at 4°C overnight (about 15 hours).

10 The resulting partially ligated plasmid was then digested with Hind III, which resulted in the loss of the polylinker region shown in Figure 6 from the Eco RI site to the Hind III site. The resulting Hind III sticky ends were then ligated to form plasmid pARC489B.

15 Positive clones were identified by plasmid DNA isolation (Maniatis), and by restriction enzyme analysis on the plasmid DNA.

In plasmid pARC489B, DNA coding for the first 13 amino acid residues of the GGPP gene was deleted. The first four amino acid residues encoded downstream from the Rec 7 promoter in plasmid pARC306A and the newly generated Eco RI blunt end were placed upstream from the former Nru I site of GGPP synthase. This altered the N-terminal amino acid sequence of GGPP synthase in the following manner. The difference in amino acid sequence became:

25 Original Amino Acid Sequence of Native Erwinia herbicola GGPP Synthase.

MET VAL SER GLY SER LYS ALA GLY VAL SER PRO HIS ARG
GLU ILE...

(SEQ ID NO:24)

30 Amino Acid Sequence of modified GGPP Synthase Gene in Plasmid pARC489B

MET ALA GLU PHE GLU ILE...
(SEQ ID NO:25)

-79-

The determined DNA sequence for this heterologous gene is illustrated in Figure 3 from about position 150 to about position 1153.

5 E. coli cells transformed with the plasmid pARC489B were assayed for GGPP synthase activity. The level of activity was found to be 12.15 nmol/min/mg protein.

10 When the plasmid pARC489B was transferred to E. coli cells that contained a plasmid containing the rest of the genes coding for enzymes required for carotenoid production, plasmid pARC808, the cells produced carotenoids. Therefore, this construction coded for an active enzyme even though the heterologous gene portion from plasmid pARC306A encoded the first
15 four amino acid residues, and the first 13 amino acid residues encoded by the gene for GGPP synthase were deleted.

20 The above described DNA segment of plasmid pARC489B overlaps bases encoding four amino acids adjacent to the Rec 7 promoter at its 5' end and extends to the blunted, former Eco RI site in the polylinker region of the plasmid. This DNA segment can be excised by reaction with Nco I at its 5' end and the Hind III or Pvu II sites as are illustrated for plasmid
25 pARC306A in Figure 6.

30 The desired GGPP synthase gene does not contain a Pvu II or a Hind III restriction site. The region between the Hind III and Pvu II sites of plasmid pARC489B contains stop codons in all three reading frames. It is preferred to utilize the Pvu II site for cleavage of the 3' end of the DNA. Thus, the desired GGPP synthase DNA segment can be referred to as lying within the approximately 1150 bp sequence between the Nco I and Pvu II restriction sites of plasmid pARC489B.

-80-

Next, the 3' end of the gene for GGPP synthase was modified. This construction was made in the following manner. A Nru I (about 11187)-Bal I (about 11347 of Figure 5) double blunt end fragment was
5 inserted into a specially prepared version of the plasmid pARC306A. Thus, plasmid pARC306A was digested with Eco RI, and the resulting ends were blunted with the Klenow fragment of DNA Pol I to produce two blunt ends. The blunt ended Nru I-Bal I fragment was
10 operatively linked into the plasmid pARC306A vector to form plasmid pARC489D. This vector included all of the polylinker restriction sites of plasmid pARC306A shown in Figure 6 except the Eco RI site.

The GGPP synthase gene-containing portion of
15 the resulting plasmid pARC489D has the same 5' end as does plasmid pARC489B, but the 3' end is about 151 bp shorter than the GGPP synthase gene in plasmid pARC489B. The sequence of the heterologous GGPP synthase structural gene of plasmid pARC489D is
20 illustrated in Figure 3 from about position 150 to about position 1002, with the 5' end of this DNA being the same as that of the GGPP synthase gene present in plasmid pARC489B.

Downstream about 70 bp from the Hind III site
25 of the multiple cloning region in plasmid pARC306A is a Pvu II site. There are no Pvu II sites in the GGPP synthase gene. Therefore, the GGPP synthase structural gene can be transferred from a pARC306A-derived plasmid such as plasmid pARC489D to other plasmids as an
30 approximately 1000 bp Nco I-Pvu II fragment.

Plasmid pARC489D was transformed into E. coli.
Very surprisingly, this construction gave the highest
enzyme activity of all the different versions of the
GGPP synthase gene. This activity was an unexpectedly
35 high 23.28 nmol/min/mg protein.

-81-

When the plasmid pARC489D was introduced into E. coli cells containing the plasmid pARC808, carotenoids were synthesized.

5 A comparison of the activities of several of the previously described GGPP synthase gene constructs is shown in Table 2 below, including the activity of a related gene present inherently in R. sphaeroides 2.4.1. Those results indicate an enhancement of about 35 to about 130 times the activity of the original
10 plasmid pARC376.

Table 2
GGPP Synthase Activity of Various Gene Constructs
As Compared to R. sphaeroides

15

		Activity
<u>Constructs</u>		<u>(nmol/min/mg protein)</u>
<u>R. sphaeroides</u> 2.4.1		0.20
pARC376		0.18
20	pARC491	7.91
	pARC417BH	6.35
	pARC418BH	0
	pARC489B	12.15
	pARC489D	23.28

25

h. GGPP synthase characterization

The plasmids pARC489B and pARC489D were introduced into the E. coli strain JM101 (BRL). These
30 cells were treated with nalidixic acid to induce the Rec 7 promoter, which caused production of large amounts of the GGPP synthase enzyme. The protein extract from these cells was separated on SDS-
polyacrylamide gel electrophoresis (PAGE). Because of
35 the very large amount of GGPP synthase produced under

-82-

these conditions, it is readily identifiable by staining with Coomassie Brilliant Blue on the SDS-PAGE system. The isolated and substantially purified GGPP synthase can then be recovered from the gels by standard procedures.

The Erwinia herbicola GGPP synthase that was produced in cells containing plasmid pARC489B was a protein of the size of about 35 kilodaltons, and is thought to be the complete, native molecule, whereas the GGPP synthase that was produced in cells with plasmid pARC489D was about 33 kilodaltons. Thus, the 5' deletion of thirteen amino acid residues and then replacement with non-Erwinia herbicola sequence of four residues, coupled with the 3' deletion of the approximately 151 bp between the Bal I site and the Eco RV site produced a protein that was about 2 kilodaltons smaller, but far more active than the native molecule. The GGPP synthase structural gene present in plasmid pARC489D is the gene most preferably used for GGPP synthase in E. coli, S. cerevisiae, and higher plants.

i. Induction of Rec 7 driven protein production

The previously discussed production of GGPP synthase in E. coli using plasmids pARC417BH, pARC489B and pARC489D was carried out using the Rec 7 promoter. Phytoene synthase production in E. coli using the plasmid pARC140N discussed below was also carried out using the Rec 7 promoter. Culture conditions for growth of the transformed E. coli cells are as follows.

A single colony from a plate containing freshly (<2 days old) transformed cells was picked, grown overnight (e.g. about 15-18 hours) in M9+CAGM medium (see Table 3B hereinafter for media formulations) + 50 µg/ml ampicillin at 30°C. Cultures

-83-

of cells were grown at various temperatures from 27-37°C by diluting the cells 1:100 into fresh M9+CAGM medium and maintaining the culture at the desired temperature. Each culture was grown until it was
5 roughly one-half of the final desired density (150-180 Klett units in a shaken culture). The culture was then induced by addition of nalidixic acid to a final concentration of 50 µg/ml. Five µl of a stock solution
10 of freshly prepared 10 mg/ml nalidixic acid in 0.1N NaOH per ml of culture to be induced was used. Induction was permitted to proceed for 2-4 hours after addition of nalidixic acid.

-84-

Table 3

A. M9+CAGM MEDIUM COMPOSITION

	Component	grams/liter
5	$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	13.2
	KH_2PO_4	3.0
	NaCl	0.5
	NH_4Cl	1.0
	Casamino Acids (Difco)	10.0
10	MgSO_4	0.3
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.004
	Glucose (Shake Flask)	3.0
	Thiamine-HCl	0.025
	FeCl_3	0.0054
15	ZnSO_4	0.0004
	CoCl_2	0.0007
	Na_2MoO_4	0.0007
	CuSO_4	0.0008
	H_2BO_3	0.0002
20	MnSO_4	0.0005

B. MEDIUM FORMULATIONS

M9+CAGM Medium for Shake Flasks (1 Liter)			
25	900 ml	distilled H_2O	Autoclaved
	40 ml	25X M9 Salts	Autoclaved
	50 ml	20% (w/v) Casamino Acids	Filtered
	6.4 ml	40% (w/v) Glucose	Autoclaved
	1.2 ml	1M MgSO_4	Autoclaved
30	0.25 ml	0.1M CaCl_2	Autoclaved
	0.25 ml	0.1% (w/v) Thiamine-HCl	Filtered
	0.1 ml	10,000X Trace Minerals	Filtered
	0.1 ml	10,000X Iron Supplement	Filtered

-85-

All components should be sterilized separately, cooled to room temperature and then combined.

C. 25X M9 Salts (1 liter)

5	Component	grams
	Na ₂ HPO ₄ ·7H ₂ O	330
	KH ₂ PO ₄	75
	NH ₄ Cl	25

10 distilled H₂O to 1 Liter

D. 10,000X Trace Minerals (200 ml)

	Component	grams
	ZnSO ₄	0.8
15	CoCl ₂	1.4
	Na ₂ MoO ₄	1.4
	CuSO ₄	1.6
	H ₂ BO ₃	0.4
	MnSO ₄	1.0

20

Dissolve in 200 ml of H₂O, add 1 drop HCl (fuming), filter sterilize.

E. 10,000X Iron Supplement (200 ml)

	Component	grams
25	FeCl ₃	10.8

Dissolve in 200 ml of H₂O, add 1 drop HCl (fuming), filter sterilize.

30

Each culture was highly aerated at all times. Fifteen ml in a 250 ml sidearm flask for analytical runs were routinely used, and 330 ml in a Fernbach (2.81) flask for semi-preparative runs were routinely used.

-86-

Production of all proteins examined so far has been quite dependent on strong aeration during the induction period.

5 j. Enzyme assay

GGPP synthase was prepared in the cell cytosol as described below.

(1) Cytosol preparation

10 The growing cells were centrifuged to form a cell pellet. The cell pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 10 percent glycerol, 0.1 mM EDTA in a 15 ml plastic conical tube and vortexed with acid washed glass beads
15 (425-600 micron for yeast cells and 75-150 micron for bacteria are typically used) for 1 minute and allowed to cool in ice for 1 minute. This was repeated three times after which the homogenate was transferred to another tube and centrifuged at 17,000 x g for 60
20 minutes at 4°C. The supernatant was next centrifuged at 150,000 x g for 60 minutes at 4°C. The supernatant thus obtained was the cell cytosol.

(2) Assay for GGPP synthase

25 Cell cytosol was preincubated for 20 minutes at 4°C with 10μM epoxy-isopentenyl pyrophosphate (IPP) in order to inhibit IPP-isomerase activity. The assay mixture, containing 40 μM farnesyl pyrophosphate (FPP) and 40 μM 14C-IPP (250,000 dpm) in 10 mM Hepes buffer
30 (pH 7.0, 1 mM MgCl₂, 1 mM DTT) in a 1 ml total volume of preincubated cytosol, was incubated at 37°C for 30 minutes.

 The reaction was terminated by transferring the assay mixture to a pre-cooled (in dry ice) tube and
35 lyophilizing for 8 hours. The dry residue was

-87-

resuspended in 0.5 ml of 0.1 M glycine buffer (pH 10.4, 1 mM MgCl₂, 1 mM ZnCl₂) and treated with 25 units of alkaline phosphatase for 3 hours at 37°C. The alkaline phosphatase reaction converted the pyrophosphates to
5 their corresponding alcohols, which were extracted with hexane, evaporated to dryness under a stream of nitrogen and redissolved in 150 µl of methanol.

Seventy-five µl of this methanol solution were injected into an HPLC connected with a C-18 econosphere
10 Altech analytical column (4.6 x 250 mm, 5 micron particle size) equilibrated with 85 percent methanol:water (4:1) and 15 percent THF:CH₃CN (1:1). A linear gradient to 80 percent methanol:water (4:1) and 20 percent THF:CH₃CN (1:1) in 20 minutes at 1.5 ml/min
15 resolved the alcohols. The HPLC was connected in series with a Radiomatic flow detector, which integrated the radioactive peaks, e.g. geranylgeraniol (GGOH) peak. Specific activity was expressed in nmol GGOH formed/min/mg of protein under the given assay
20 conditions. Protein was determined by the Bradford method using BSA as the standard.

Example 3. Phytoene Synthase Gene

a. Digestion of pARC376 with Pst I

25 The plasmid pARC376-Pst 122 was created by deletion of an approximately 592 bp Pst I Erwinia herbicola DNA fragment from Pst I sites at about 5807 to about 5215 of plasmid pARC376 (Figure 5), followed by religation of the larger of the two fragments. The
30 Eco RI (about 3370) to Hind III (about 13463) fragment from plasmid pARC376-Pst 122, which contains the desired Erwinia herbicola DNA fragment, was cloned into the plasmid pARC305A, resulting in plasmid pARC139.

35 The plasmid pARC305A contains the polycloning linker from pUC18, the chloramphenicol

-88-

acetyltransferase gene (CAT) that confers chloramphenicol resistance in E. coli and the pMB1 origin of replication. The plasmid pARC305A is an analogous plasmid to plasmid pUC18 except plasmid pARC305A contains the CAT selectable marker whereas pUC18 contains the ampicillin selectable marker.

When the resulting Erwinia herbicola DNA was inserted into the plasmid pARC305A to create the plasmid pARC139 and introduced into E. coli cells, no carotenoids were made, as expected.

An impairment of the gene for phytoene synthase would cause the E. coli cells not to produce any colored carotenoids. Therefore, the deletion of this 592 bp region could have deleted part of the gene for phytoene synthase.

b. Construction of Plasmid pARC285

The construction of plasmid pARC285 used the approximately 1112 bp Nco I to Eco RI fragment from the plasmid pARC376-Bam 100. The plasmid pARC376-Bam 100 is a derivative of the pARC376 plasmid in which the approximately 1045 bp Bam HI fragment from about position 3442 to about position 4482 (Figure 5) and the approximately 815 bp Bam HI fragment from about position 4487 to about 5302 (Figure 5) were deleted. A total of about 1860 nucleotides was deleted from the pARC376 plasmid. As a result of the deletions of the Bam HI fragments from plasmid pARC376, the Bam HI site at about 5354 at the 3' end was brought within about 72 nucleotides of the Eco RI site originally at about position 3370 of plasmid pARC376. The resulting restriction fragment therefore contained about 1112 bp and was bounded by Nco I and Eco RI restriction sites at its 5' and 3' ends, respectively.

-89-

The phytoene synthase gene is contained on an approximately 1040 bp Nco I to Bam HI restriction fragment (corresponding approximately to positions 6342 and 5302 of Figure 5, respectively), but it can be
5 cloned into other plasmids as an approximately 1112 bp Nco I to Eco RI fragment. The approximately 1112 bp Nco I to Eco RI fragment was excised from the plasmid pARC376-Bam 100 and cloned into the Nco I to Eco RI sites of plasmid pARC306A to generate plasmid pARC285.
10 The relevant portion of the phytoene synthase gene can thus be excised from plasmid pARC285 as an approximately 1112 bp Nco I to Eco RI fragment.

c. Construction of Plasmid pARC140N

15 Analysis of the region surrounding the Nco I (about position 6342) site revealed that the methionine codon internal to the Nco I site was in an open reading frame that had another methionine codon 13 amino acid residues upstream. Immediately upstream from this
20 methionine codon, was a consensus sequence for the ribosome binding site (AGGA) that is often found in procaryotic organisms upstream from the initiation codon of a gene.

To determine if the upstream methionine was in
25 fact the initiation codon, a Bgl II site was introduced immediately upstream from the methionine codon of the Nco I site, using in vitro mutagenesis, as described before. Two complementary polynucleotide sequences were made that contained a Nco I overhang on one end
30 and on the other end a Bgl II overhang. The sequences were as follows:

-90-

Bgl II

Nco I

5'

(SEQ ID NO:26) 3'

GATCTAAAATGAGCCAACCGCCGCTGCTTGACCACGCCACGCAGAC

ATTTTACTCGGTTGGCGGCGACGAACTGGTGCGGTGCGTCTGGTAC

5

3'

(SEQ ID NO:27) 5'

The two complementary single stranded polynucleotide sequences were hybridized together, ligated to an approximately 1112 bp Nco I-Eco RI fragment from plasmid pARC285 containing the approximately 1040 bp Nco I to Bam HI phytoene synthase gene region and cloned into plasmid pARC135.

The plasmid pARC135 (shown in Figure 7) is composed of the pUC18 vector containing the yeast PGK promoter and terminator sequences separated by a unique Bgl II site.

First, the approximately 3.1 kb Hind III fragment of yeast (*S. cerevisiae*) containing the PGK gene was cloned into the Hind III site of pUC18 to create plasmid pSOC117 (also referred to herein as pARC117). Next, a Bgl II site was introduced by oligonucleotide mutagenesis upstream of the initiating ATG codon of the PGK gene contained within a mp19M13 clone, producing the change shown below in bold.

Native PGK Sequence: Met Ser Leu
ACAACAAAATATAAAACA ATG TCT TTA
 (SEQ ID NO:28)

New PGK Sequence:
ACAACA**GATCT**ATAAAACA ATG TCT TTA
 (SEQ ID NO:29)

Bgl II Site

Then, an approximately 1.1 kb Bst XI fragment, carrying the introduced Bgl II PGK site, was excised from the mp19 clone and used to replace the homologous Bst XI fragment within plasmid pSOC117. Finally, the Bgl II fragment, containing the majority of the PGK structural

-91-

gene, was removed by Bgl II digestion, and the plasmid was religated to yield plasmid pARC135. Plasmid pARC135 was digested with Nco I and Eco RI, the resulting gene was thereafter manipulated, as discussed below, to generate the plasmid pARC140R, which contains the S. cerevisiae phosphoglyceric acid kinase (PGK) promoter at the Bgl II site.

The experimental protocol for the construction of plasmid pARC140R is described below.

10 A. Hybridization/Annealing of the two

oligonucleotide probes (oligonucleotide probes were not phosphorylated at the 5' end).

1) The two complementary oligonucleotide probes were annealed in 25 μ l of solution containing:

15 10 μ l of oligonucleotide #1 (about 1 μ g)
10 μ l of oligonucleotide #2 (about 1 μ g)
1.65 μ l of 1 M Tris-Cl₂ (pH 8.0)
2.5 μ l of 100 mM MgCl₂
20 0.45 μ l water

2) The probe solution was incubated at 65°C for 10 minutes. Then it was cooled according to the following regime:
20 minutes at 55°C
25 20 minutes at 42°C
20 minutes at 37°C
30 minutes at room temperature (24°C)

B. An approximately 1112 bp fragment from Nco I to Eco RI in plasmid pARC285, containing an approximately 1040 bp (Nco I to Bam HI) sequence was excised and isolated from the gel. This approximately 1112 bp fragment contained the shortened version of the gene for phytoene synthase.

-92-

C. The annealed oligonucleotide probes were ligated overnight (15 hours at 15°C) to the approximately 1113 bp (Nco I to Eco RI) fragment according to the following protocol:

5

Annealed oligos	25 μ l
Nco I-Eco RI fragment	20 μ l (about 1 μ g)
10 X Ligation Buffer	5 μ l (IBI, Corp.)
T4 Ligase (Boehringer-Mannheim)	

10 The result from the ligation was the following:

Bgl II	Nco I		Bam HI	Eco RI
I-----I	-----I	-----I	-----I	-----I

15 D. The mixture was subsequently phenol extracted, chloroform: isoamyl alcohol (24:1) extracted and then ethanol precipitated. The DNA pellet was resuspended in 27 μ l water.

20 E. The DNA pellet was then digested for 30 minutes at 37°C with Eco RI to remove any dimers that may have formed during the ligations.

DNA fragment	27 μ l
Eco RI digestion buffer (BRL)	3 μ l
Eco RI enzyme (BRL)	3 μ l (30 U)

25 F. The products of the Eco RI digestion were separated by electrophoresis on a 0.7 percent agarose gel. The fragment (about 1158 bp) was isolated from the gel.

30 G. This Bgl II to Eco RI fragment was cloned into the Bgl II and Eco RI sites of the plasmid pARC135 as follows. About 5 μ g of plasmid pARC135 was digested with Bgl II and Eco RI and then separated on a 0.7 percent agarose gel. A DNA fragment (about 4 kb) was isolated. The
35 approximately 1158 bp Bgl II to Eco RI fragment

-93-

containing the full length phytoene synthase gene was cloned into the approximately 4 kb vector in the Bgl II and Eco RI sites according to the following protocol:

5

pARC135 Bgl II/Eco RI digested	10 μ l (about 0.2 μ g)
Bgl II to Eco RI fragment	20 μ l (about 0.5 μ g)
10 X ligation buffer	3 μ l
T4 ligase	2 μ l (4 Units)

10 The reaction was incubated overnight (about 15-18 hours) at 15°C.

H. The ligated DNA was cloned into DH5-alpha E. coli cells obtained from BRL.

15 I. Transformants were grown in the presence of 100 μ g/ml of ampicillin. Colonies containing the cloned DNA fragment were identified by growing prospective clones in the presence of ampicillin, isolating plasmid DNA by the alkali lysis procedure and performing restriction
20 enzyme analysis on the clones. The result of this cloning procedure was a plasmid named pARC140R that contained the desired genes.

25 Upstream from the ATG methionine codon, three adenine residues were introduced. Presence of adenine residues adjacent to the initiation codon has been correlated with genes that are highly expressed in S. cerevisiae. These residues had been inserted in the sequence to cause high level expression of a gene in
30 S. cerevisiae (Hamilton et al., Nucleic Acids Research, 15:3581 1987). The plasmid pARC140R contains the S. cerevisiae promoter from the gene for phosphoglyceric acid kinase (PGK) adjacent to the gene for phytoene synthase.

-94-

The modified phytoene synthase structural gene was excised from plasmid pARC140R as an approximately 1158 bp Bgl II-Eco RI fragment, engineered and cloned into plasmid pARC306N to generate plasmid pARC140N.

5 The plasmid pARC306N is similar to plasmid pARC306A except that instead of an Nco I site adjacent to the E. coli Rec 7 promoter, there is an Nde I site.

More specifically, plasmid pARC306N was digested with Nde I and then digested with S1 nuclease
10 to blunt the ends of the former Nde I sites. The plasmid was thereafter digested with Eco RI to remove one of the blunt ends and provide an Eco RI sticky end.

Plasmid pARC140R was digested with Bgl II and then with S1 nuclease to blunt the resulting ends. The
15 digested and blunt-ended plasmid was then further digested with Eco RI to remove one of the blunt ends and provide an Eco RI sticky end for the DNA containing the phytoene synthase structural gene. That structural gene was therefore in a fragment of about 1164 bp with
20 a blunt end at one end and an Eco RI site at the other end.

The above phytoene synthase structural gene-containing DNA segment was ligated into the blunt end and to Eco RI portions of the above-digested plasmid
25 pARC306N to operatively link the two DNA segments together and form plasmid pARC140N. The phytoene synthase structural gene-containing DNA segment can be excised from plasmid pARC140N as an approximately 1176 bp Hpa I-Eco RI fragment, an approximately 1238 bp Pvu
30 II-Eco RI fragment or as a still larger fragment using one of the restriction sites in the polylinker region downstream from the Eco RI site (see, Figure 6).

The plasmid pARC140N, was transferred into
35 E. coli cells that contained the plasmid pARC139, in which part of the gene for phytoene synthase was

-95-

deleted and, those E. coli cells were therefore incapable of producing any colored carotenoids. When plasmid pARC140N was added to those E. coli cells containing plasmid pARC139, the cells produced colored carotenoids. This demonstrated that the modified gene for phytoene synthase coded for a functional enzyme.

E. coli cells containing plasmid pARC140N were induced with nalidixic acid to produce large amounts of the phytoene synthase protein according to the protocol discussed hereinbefore. The protein fraction was isolated and analyzed by SDS-PAGE and revealed that the size of phytoene synthase protein is 38 kilodaltons.

Example 4. Phytoene Production in E. coli

a. Method One - Plasmid containing the engineered genes for GGPP synthase and phytoene synthase

A plasmid containing genes for both GGPP synthase and phytoene synthase, as well as an associated promoter regulatory region adjacent to a structural gene causes E. coli cells containing this plasmid to produce phytoene. An example of such a plasmid construct is the use of the structural gene for GGPP synthase from the plasmid pARC489D with a promoter that functions in E. coli adjacent to the 5' end of the structural gene for GGPP synthase. This construct is introduced into a common cloning vector such as pUC18. Where the structural genes are linked together, a single promoter can function in E. coli to express both gene products.

A before-described structural gene for phytoene synthase excised from the plasmid pARC140R is cloned adjacent to a promoter that functions in E. coli, such as Rec 7. This Rec 7 promoter-phytoene synthase heterologous gene is then introduced into a plasmid containing the gene for GGPP synthase. The

-96-

plasmid containing both of these genes directs phytoene synthesis in E. coli. The two genes can also be placed end-to-end in E. coli under the control of a single promoter.

5

b. Method Two - Plasmid pARC376 with a defective gene for phytoene dehydrogenase-4H

Phytoene production can occur with the native pARC376 plasmid in which the genes for GGPP synthase and phytoene synthase are functional and produce functional proteins, but in which the gene for phytoene dehydrogenase-4H is impaired, thereby impairing the production of lycopene from phytoene. A plasmid pARC376 derivative in which the gene for phytoene dehydrogenase-4H is deleted or in some other way impaired could not further metabolize the phytoene being produced in the E. coli cells due to the action of the genes for GGPP synthase and phytoene synthase. Under this condition, phytoene accumulates. The gene for phytoene dehydrogenase-4H is located approximately between the positions 7849 to 6380 of plasmid pARC376 as shown in Figure 5.

By example, two different pARC376 derivative plasmids that contain deletions at the beginning of the gene for phytoene dehydrogenase-4H have been made as described before. One plasmid is pARC376-Bam 127, in which the approximately 2749 bp Bam HI fragment from about position 7775 to about 10524 (Figure 5) was deleted. The other was plasmid pARC376-Pst 110 missing a Pst fragment at 7792-10791 (Figure 5). These plasmids were constructed by partially digesting plasmid pARC376 with either Bam HI or Pst I, and ligating the respective DNA fragments together.

These deletions caused the gene for phytoene dehydrogenase-4H to be non-functional, since the

-97-

beginning part of the gene was deleted. E. coli cells that contained either plasmid pARC376-Bam 127 or plasmid pARC376-Pst 110 produce phytoene. Phytoene is colorless and cells that produce phytoene have the same colorless character as normal E. coli cells. The ligation mixture was transformed into E. coli and any resulting colorless colonies were analyzed for the presence of phytoene. The presence of phytoene was confirmed by growing E. coli cells containing the plasmid, performing an extraction according to the following protocol, and identifying phytoene by HPLC analysis in the extract.

c. Identification of Phytoene Produced by Transformed E. coli

i. Extraction from cells

One hundred to 500 mg of lyophilized E. coli cells containing an above-described plasmid were resuspended in 3 ml of 7:2 acetone:methanol in 15 ml conical glass tube with teflon seal cap. 450-600 Micron glass beads (1:1 ratio with the cells) were added to the tube, which was covered with foil and vortexed for 2 minutes. After 5 minutes, the tube was spun in a table top centrifuge and the supernatant transferred to a foil covered glass vial. This extraction was repeated multiple times.

The entire pool of the extract was filtered through a 0.2 micron Acrodisc CR filter in a glass syringe, and the filtrate was dried under nitrogen. Utmost care was taken to protect the carotenoids/xanthophylls from light and heat.

ii. Identification

The presence of phytoene was monitored by thin layer chromatography (TLC) analysis in three different

-98-

solvent systems using authentic phytoene as a reference.

5 The carotenoids/xanthophylls were separated by
high pressure liquid chromatography (HPLC) with the aid
of a Hewlett Packard C-18 Vydac analytical column (4.6
x 250 mm, 5 micron particle size). A linear gradient
from 30 percent isopropanol and 70 percent
acetonitrile:water (9:1) to 55 percent isopropanol and
45 percent acetonitrile:water (9:1) in 30 minutes (min)
10 at 1 ml/min resolved most of the compounds of interest
with the following retention times - zeaxanthin 8.7
min, lycopene 16.2 min, beta-carotene 18.1 min,
phytofluene 19.9 min, phytoene 21.8 min, and the
zeaxanthin diglucosides were clustered between 6 and 8
15 min.

The amount of phytoene produced in these cells
averaged about 0.01 percent (dry weight).

Example 5. Phytoene Production in *S. cerevisiae*

20 *S. cerevisiae* does not normally produce
carotenoids since it does not have the necessary
functional genes for phytoene production.

S. cerevisiae does, however, produce farnesyl
pyrophosphate (FPP). For phytoene production to occur
25 in *S. cerevisiae*, the genes for GGPP synthase and
phytoene synthase need to be transferred into the *S.*
cerevisiae cells in the proper orientation to permit
the expression of functional enzymes.

Promoter sequences that function in
30 *S. cerevisiae* need to be placed adjacent to the 5' end
of the structural genes for GGPP synthase and phytoene
synthase and termination sequences can also be placed
at the 3' ends of the genes. The genes for GGPP
synthase and phytoene synthase that contain the proper

-99-

regulatory sequences for expression in S. cerevisiae then are transferred to the S. cerevisiae cells.

a. Construction of pARC145B

5 The vector pSOC713 (Figure 8), was made by
first using Klenow polymerase to make blunt ends on the
Eco RI fragment of the yeast B-form 2-micron circle
that contains the 2-micron origin of replication.
Thus, the blunt-ended fragment was cloned into the Sma
10 I site of pUC8. The 2-micron fragment was removed from
the pUC8 construct by cleavage with Eco RI and Bam HI.
This Eco RI-Bam HI fragment was ligated to the Eco RI-
Bgl II fragment of yeast DNA which contains the TRP 1
gene. The DNA containing the fused TRP 1 to 2-micron
15 fragment was ligated as an Eco RI fragment into the Eco
RI site of pUC18. Finally, a region of the yeast
genome, containing the divergently-facing GAL 10 and
GAL 1 promoters was ligated as an Eco RI to Bam HI
fragment into the above TRP 1/2-micron/pUC18 plasmid,
20 which had been cleaved with Eco RI and Bam HI. The
restriction map of plasmid pSOC713 is shown in
Figure 8.

Three modifications were made to plasmid
pSOC713 to yield plasmid pARC145B (Figure 9). First,
25 plasmid pSOC713 was partially digested with Eco RI and
the ends were made blunt with Klenow polymerase and
self-ligated. The resultant plasmid contained a unique
Eco RI site adjacent to the GAL 1 promoter region.
This plasmid was cleaved with Eco RI and the synthetic
30 oligonucleotide shown below,

5' AATTCCTGGGCCATGGC 3' (SEQ ID NO:30)
3' GGGCCCGGTACCGTTAA 5' (SEQ ID NO:31)

was ligated into the Eco RI site. This regenerated one
35 Eco RI site followed by Sma I and Nco I sites.
Finally, the single Bam HI site was cut, filled in with

-100-

Klenow polymerase, and the Bgl II synthetic linker oligonucleotide

CAGATCTG
GTCTACTG

5

was ligated, cut with Bgl II, and then self-ligated to make a Bgl II site flanked by two Bam HI sites. The restriction map of plasmid pARC145B is shown in Figure 9.

10

b. Construction of Plasmid pARC145G

The engineered gene for GGPP synthase contained in plasmid pARC489D, which encoded the most active version of the enzyme in E. coli above, was transferred to the S. cerevisiae vector pARC145B to generate plasmid pARC145F. This was accomplished by digestion of plasmid pARC489D with Nco I and Pvu II to obtain the approximately 1000 bp Nco I-Pvu II restriction fragment that contained the GGPP synthase structural gene. An Nco I linker was added to the Pvu II site of the restriction fragment to make that fragment an Nco I-Nco I fragment containing about 1010 bp. The GGPP synthase gene was cloned adjacent to the S. cerevisiae divergent promoter region GAL 10 and GAL 1 so that the GGPP synthase gene would be expressed in S. cerevisiae using the GAL 10 promoter.

25

The gene for phytoene synthase from plasmid pARC140R (Example 2) was excised and placed adjacent to the other side of the GAL 1 promoter of plasmid pARC145F so that the phytoene synthase gene would also be expressed using the GAL 1 promoter. Thus, the transcription termination sequence from the S. cerevisiae gene PGK was cloned at the 3' end of the gene for phytoene synthase.

30

35

More specifically, plasmid pARC145F was digested with Bgl II and Sph I, whose restriction sites

-101-

are illustrated in Figure 9 for precursor plasmid pARC145B. The phytoene synthase structural gene was excised from plasmid pARC140R as an approximately 1158 Bgl II-Eco RI fragment; the same structural gene is present in the approximately 1176 bp Hpa I-Eco RI fragment of plasmid pARC140N. The approximately 500 bp PGK termination sequence from another plasmid, pARC117, was excised as an Eco RI-Sph I fragment such as the same fragment shown in plasmid pARC135 of Figure 7. The Bgl II-Sph I digested plasmid pARC145F, the Bgl II-Eco RI about 1158 bp plasmid pARC140R fragment and the about 500 bp Eco RI-Sph I PGK termination sequence were triligated to operatively link the three sequences together.

This ligation placed the phytoene synthase structural gene adjacent to and under the control of the GAL 1 promoter at the 5' end of the structural gene. The PGK termination sequence was placed at the 3' end of the phytoene synthase structural gene. The resulting plasmid, now containing both of the genes required for phytoene production under control of the GAL 10 and GAL 1 divergent promoters, was named plasmid pARC145G, and is shown in Figure 10. Other relevant features of plasmid pARC145G include the 2 micron origin of replication of S. cerevisiae and the TRP 1 gene of S. cerevisiae as a selectable marker.

The plasmid pARC145G was transferred into the S. cerevisiae strain YPH499 (provided by Dr. Phillip Heiter, Johns Hopkins University) that lacked a functional TRP 1 gene. This strain was able to utilize galactose as a carbon source. Transformants were isolated, and the cells were grown in the presence of galactose to induce the GAL 10 and GAL 1 promoters to express the genes for phytoene production.

-102-

The S. cerevisiae cells were grown on the media described below to produce phytoene. YPH499 is a strain of yeast that contains an impaired TRP 1 gene and an impaired URA 3 gene, and is able to utilize galactose as carbon and energy sources. This strain requires tryptophan and uracil in the growth medium in order to grow. Alternatively, these strains can be grown if they are transformed with a plasmid (or plasmids) containing a normal copy of either the TRP 1 gene, but not a normal copy of the URA 3 gene, in which case the cells require uracil to be added to the growth medium, or the URA 3 gene, but not a normal copy of the TRP 1 gene, in which case the cells need to have tryptophan added to the growth medium.

There are four different media used to grow this strain of Saccharomyces:

Medium 1 is used if the cells contain no further URA 3 or TRP 1 genes.

Medium 2 is used if the cells contain a plasmid(s) with only the TRP 1 gene.

Medium 3 is used if the cells contain a plasmid(s) with only the URA 3 gene.

Medium 4 is used if the cells contain a plasmid(s) with both the TRP 1 and the URA 3 genes.

The media constituents are as follows:

Basic Constituents:

0.67% Yeast Nitrogen Base without Amino

Acids (Source Difco, #0919-15);

2% Galactose; and

720 mg/l Dropout Mixture*

* Dropout Mixtures

-103-

For Medium 1 (Complete)

	<u>Constituent</u>	<u>Amount (mg)</u>
	adenine	400
	uracil	400
5	tryptophan	400
	histidine	400
	arginine	400
	methionine	400
	tyrosine	600
10	leucine	1200
	lysine	600
	phenylalanine	1000
	threonine	4000
	aspartic acid	2000
15	For Medium 2, without the tryptophan.	
	For Medium 3, without the uracil.	
	For Medium 4, without both tryptophan and uracil.	

20 To prepare a dropout mixture all of the desired constituents were added to a mortar and ground thoroughly with a pestle. The constituents were thoroughly mixed and 720 mg of the dropout mixture were added for each liter of medium.

25 The plasmid pARC145G contains both the GGPP synthase and phytoene synthase genes and a normal copy of the TRP 1 gene. Saccharomyces cells containing pARC145G were grown in Medium 2 with 2 percent galactose.

30 The S. cerevisiae cells were analyzed for the presence of phytoene. A total of 0.12 percent (dry weight) phytoene and related compounds having superimposable UV-Vis spectra as phytoene was found in the cells.

35

-104-

Example 6. Phytoene Production in Higher Plants

To transfer the genes for GGPP synthase and phytoene synthase to plants, the Agrobacterium gene transfer system is preferably used. The structural gene for GGPP synthase discussed before is introduced into the plasmid pCaMVCN (Pharmacia), by replacing the structural gene for chloramphenicol acetyltransferase (CAT) with the gene for GGPP synthase. The Erwinia herbicola GGPP synthase gene is preferably derived from the E. coli plasmid, pARC489D, described above, although another Erwinia herbicola GGPP structural gene can be used.

The GGPP synthase gene in pCaMVCN is adjacent to the CaMV 35S promoter, and the NOS polyadenylation site is at the 3' end of the GGPP synthase gene. This gene construct is transferred to the plasmid pGA482 (Pharmacia). The relevant features of the resulting plasmid are that (i) it contains an origin of replication that permits it to be maintained in Agrobacterium tumefaciens, (ii) there is a NOS promoter adjacent to the kanamycin resistance gene that confers kanamycin resistance to plant cells, (iii) there is a polycloning site to introduce desired genes to be transferred to plants, and (iv) the border sequences from the Agrobacterium tumefaciens T-DNA direct the integration of the desired genes into the plant genome.

The gene for phytoene synthase from plasmids pARC140R, pARC140N or another, previously described phytoene synthase structural gene is transferred to pCaMVCN, replacing the CAT gene. This gene is adjacent to the CaMV 35S promoter, and the NOS polyadenylation site is at the 3' end of the phytoene synthase gene. This gene is transferred to the pGA482 derivative that already contains the gene for GGPP synthase. The result is a gene construct in which both the genes for

-105-

GGPP synthase and phytoene synthase can be expressed in plants.

This vector is transferred to a suitable Agrobacterium tumefaciens strain such as A281 (Pharmacia) or LBA4404 (Clontech). It is noted that the previously discussed results with A. tumefaciens, Example 1b, illustrate successful introduction of genes for not only phytoene, but also for additional enzymes in the carotenoid pathway, the successful expression of the enzymes, and production of phytoene and other carotenoids in those bacteria.

Subsequently, the genes for GGPP synthase and phytoene synthase are transferred to the plant genome after the Agrobacterium tumefaciens cells infect the plant cells. Suitable plants include tobacco and alfalfa, although others could be used. The genes are expressed in the growing plant, using the CaMV 35S promoter, and the enzymes are deposited in the cytoplasm. Thus, phytoene is produced in the cytoplasm from the engineered enzymes and the naturally occurring, ubiquitous precursors.

Example 7. Phytoene Production in Higher Plant Chloroplasts

To target the GGPP synthase and phytoene synthase enzymes to the chloroplast, a DNA sequence that encodes a transit peptide sequence, which directs proteins to the chloroplast, is introduced in frame at the beginning of the genes for these two enzymes. The order of the gene construction, then, is (i) a promoter that functions in plants, (ii) the DNA sequence for the transit peptide, (iii) the carotenoid structural gene, and (iv) a plant polyadenylation sequence. An exemplary system is discussed below.

-106-

The DNA sequence for the transit peptide from the "Small Subunit" of the enzyme ribulose biphosphate carboxylase from tobacco are synthesized from oligonucleotide probes. A Nco I site is placed at the 5' end of the transit peptide sequence, at the initiation methionine codon. A Sph I site is placed at the 3' end of the transit peptide sequence. Details relating to this transit peptide, its gene and the use of its gene are in Example 14.

The carotenoid genes for GGPP synthase and phytoene synthase are fused in-frame at the Sph I site of the transit peptide sequence. The chimeric gene is cloned into the plasmid pCaMVCN, replacing the CAT gene.

The result of these constructions is a DNA segment comprising, (i) the plant CaMV 35S promoter adjacent to the transit peptide sequence, followed by (ii) a structural gene for either GGPP synthase or phytoene synthase, or both, and followed by (iii) the NOS polyadenylation site. This gene construct is transferred to the plasmid pGA482.

The pGA482 plasmid, containing the genes for GGPP synthase and phytoene synthase, is transferred to A. tumefaciens. The genes for GGPP synthase and phytoene synthase are transferred into plants following infection of the plant tissue with the Agrobacterium strain.

Suitable host plants include tobacco and alfalfa and others. The genes are expressed from the CaMV 35S promoter, the protein is directed to the chloroplast by the presence of the transit peptide sequence, and the enzyme is delivered inside the chloroplast. Through the action of the engineered Erwinia herbicola genes for GGPP synthase and phytoene

-107-

synthase, and the ubiquitous precursors, phytoene is accumulated.

Example 8. Phytoene Dehydrogenase-4H Gene

5 **a. Localization**

 The gene for phytoene dehydrogenase-4H is found on the plasmid pARC376. The general region of its location on this plasmid was shown by deleting specific regions of the pARC376 plasmid and analyzing
10 the carotenoids produced. When an altered or mutated phytoene dehydrogenase-4H gene is generated, the phytoene that is produced by the presence of the two enzymes GGPP synthase and phytoene synthase would accumulate.

15 The pARC376 plasmid (Figure 5) was partially digested with either Bam HI or Pst I restriction enzymes, and the free ends were ligated together. This DNA was transformed into E. coli HB101, and colorless colonies were picked and analyzed for the presence of
20 phytoene. Two different plasmid deletions caused the E. coli cells to accumulate phytoene, including plasmid pARC376-Bam 127, which had a 2749 bp Bam HI fragment (7745-10524) deletion and plasmid pARC376-Pst 110, which had a 2999 bp Pst I fragment (7792-10791)
25 deletion.

 The plasmid pARC376-Pst 110 was constructed as follows. Plasmid pARC376 was partially digested with Pst I, the DNA was ligated, the ligation mixture was transformed into E. coli HB101, and the cells were
30 grown in Luria-Broth supplemented with 100 µg/ml ampicillin. The transformants were screened by isolating plasmid DNA and performing restriction enzyme analysis. A plasmid with only the 2999 bp Pst I segment deleted, was identified and named pARC376-Pst

-108-

110. This deletion involves the beginning sequence of the gene for phytoene dehydrogenase-4H.

5 In E. coli cells containing either of these above two plasmids, phytoene accumulated to about 0.02 percent dry weight. This indicated that the gene for phytoene dehydrogenase-4H was present somewhere in the deleted region.

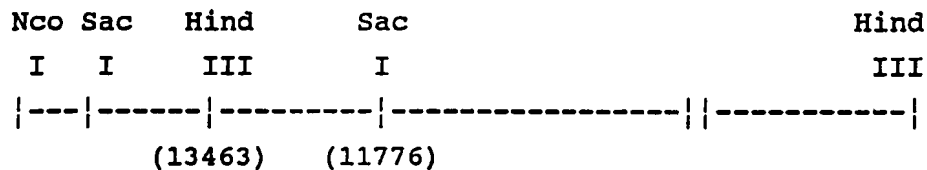
b. Construction of the plasmid pARC136

10 An about 12,000 bp Eco RI fragment from plasmid pARC376 was obtained by removal of the segment from about position 3370 to about position 379 (Figure 5). The resulting large fragment containing all of the Erwinia herbicola carotenoid genes, was inserted into
15 the Eco RI site of the pBluescript SK + plasmid (Stratagene, Inc., San Diego) resulting in plasmid pARC176B. Adjacent to the Eco RI site on the pBluescript plasmid is a Hind III site. There is another Hind III site in the insert from plasmid
20 pARC376 (position 13463).

The plasmid pARC176B was digested with Hind III, releasing an about 10,200 bp fragment that contains all of the carotenoid genes. This fragment was cloned into the Hind III site of the plasmid
25 pARC306A (described before and shown in Figure 6). The resulting plasmid was named pARC137B.

There are two Sac I sites in the plasmid pARC137B; one in the polylinker from plasmid pARC306A, the other in the GGPP synthase structural gene at about
30 position 11776 (Figure 5). Diagrammatically, the orientation is as follows:

-109-



5

The plasmid pARC137B was digested with Sac I, deleting a 1700 bp Sac I fragment from the Sac I site in the polylinker to the Sac I site at position 11776. The remaining large DNA fragment was ligated together, forming plasmid pARC136, which was transformed into E. coli HB101, and grown in Luria-Broth supplemented with 100 µg/ml of ampicillin.

E. coli cells containing plasmid pARC136 were treated with nalidixic acid to induce the Rec 7 promoter (as described before). One of the proteins produced was a 51 kilodalton protein, which upon examination by polyacrylamide gel electrophoresis (PAGE) was determined to be the phytoene dehydrogenase-4H enzyme.

This protein was electroeluted and subjected to N-terminal amino acid sequencing to obtain the sequence of the first 30 amino acid residues. Comparison of the determined amino acid sequence of this 51 kilodalton protein with the DNA sequence of plasmid pARC376 indicated that the initiation site of the phytoene dehydrogenase-4H structural gene is located at about position 7849 of plasmid pARC376 (Figure 5).

The 3' end of the phytoene dehydrogenase-4H gene extends beyond the Bgl II site at position 6836 (Figure 5). The Bgl II site of the insert to plasmid pARC136 was digested and the ends were polished with the Klenow fragment of DNA Polymerase I, religated and transformed into E. coli cells. These manipulations caused an inhibition of phytoene dehydrogenase-4H and caused the E. coli cells to accumulate phytoene,

indicating that the 3' end of the phytoene dehydrogenase-4H structural gene is downstream from the Bgl II site.

c. Construction of the plasmid pARC496A

The plasmid pARC376 was digested with Sal I restriction enzyme to excise two adjacent DNA segments; an about 1092 bp Sal I segment (positions 9340-10432 of Figure 5), and an about 3831 bp Sal I segment (positions 10432-14263 of Figure 5). The free ends of the remaining DNA fragment were religated to form the plasmid, pARC271D.

To introduce a Nco I site at the initiation methionine of the structural gene for phytoene dehydrogenase-4H, an about 3035 bp Sal I (9340) to Xmn I (6305 of Figure 5) fragment was excised from plasmid pARC271D. This fragment was isolated on agarose gel electrophoresis and used as the template for polymerase chain reaction (PCR). The following oligonucleotide probe was used:

5' AAA Nco I CCA TGG AAA AAA CCG TTG TGA TTG GC 3'
(SEQ ID NO:32)

For the PCR to run properly, the 3' end must also be amplified in order to make the proper strands of the DNA fragment desired. The 3' end of the second strand oligonucleotide probe retaining the native DNA sequence was:

Nco I (Nco I site at position 6342 of Figure 5)
5' GG C CAT GG T CTG CGT GGC GTG 3'
(SEQ ID NO:33)

d. PCR Reaction:

The GeneAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus) was used to perform the reaction. The following components were mixed in the

-111-

quantity and order specified according to the manufacturers instructions.

	<u>Component</u>	<u>Order of Addition</u>	<u>Volume</u> (μ l)
5	Final		
	Concentration		
10	Sterile Water	1	43.5
	10 X Rxn. Buffer	2	10 1 X
	1.25 mM dNTP Mix	3	16 200 μ M dNTP
	Primer 1 (10 pMole/ μ l)	4	10 1 μ M
	Primer 2 (10 pMole/ μ l)	5	10 1 μ M
15	Template DNA	6	10 100 ng
	Taq Polymerase	7	0.5 2.5 Units

100 μ l of mineral oil was layered on top of the reaction mixture, and the reaction was performed using the Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer, Prairie Cloud, MN). The method consisted of 25 cycles of amplification. One cycle included the following:

- 1) 1 minute denaturation at 92°C;
- 2) 2 minute template priming at 37°C;
- 3) 3 minute polymerization at 72°C; and
- 4) 7 minute polymerization at 72°C.

After the reaction was completed the mineral oil was removed, the reaction mixture was extracted twice with ether, and the DNA was precipitated with ethanol.

e. Cloning of the PCR produced DNA fragment

- 1) The DNA produced by the PCR reaction was digested with Nco I. This produced a DNA fragment of about 1509 bp, which was isolated and recovered from an agarose gel.

-112-

- 2) About 5 μ g of the plasmid pARC306A was digested with Nco I.
- 3) About 100 ng of the Nco I-digested plasmid pARC306A was admixed with about 200 ng of the Nco I fragment produced by the PCR reaction. The fragments were inserted using ligation buffer (2 μ l) (IBI Corp.) and 1 Unit of T4 ligase in a total volume of 20 μ l. The ligation reaction was incubated at 4°C for about 15 hours.
- 4) The ligation mixture was transformed into E. coli HB101. Transformants were selected on Luria-Broth with 100 μ g/ml ampicillin. DNA was isolated from prospective clones and the clone carrying the phytoene dehydrogenase-4H gene insert was identified by restriction enzyme analysis. This plasmid was named pARC496A.

The DNA sequence for the phytoene dehydrogenase-4H gene was determined as described before and is shown in Figure 11, along with some of the restriction sites. The approximately 1505 bp Nco I-Nco I fragment (Nco I fragment) present in plasmid pARC496A is a particularly preferred DNA segment herein.

f. Proof of a Functional Genetically Engineered Phytoene Dehydrogenase-4H Gene

The proper functioning of the gene for phytoene dehydrogenase-4H in plasmid pARC496A was established by complementation of the plasmid pARC275 (described in Example 9). This plasmid has three relevant features: 1) it is a derivative of plasmid pARC376 in which part of the gene for phytoene dehydrogenase-4H gene has been deleted, therefore, the plasmid causes the accumulation of phytoene in E.

-113-

coli; ii) it contains the R1162 origin of replication; and iii) it contains a kanamycin resistance gene from Tn5, and therefore, E. coli cells that contain plasmid pARC275 are able to grow in the presence of 25 µg/ml kanamycin.

E. coli cells containing pARC275 were transformed with the plasmid pARC496A to form doubly transformed host cells. These host cells were grown in medium supplemented with 25 µg/ml kanamycin and 100 µg/ml of ampicillin. The cells produced lycopene at a level of about 0.01 percent dry weight.

This result demonstrated that the gene for phytoene dehydrogenase-4H had been successfully engineered. In addition, this result showed that the approximately 1505 bp Nco I-Nco I DNA segment present in plasmid pARC496A contained the entire DNA sequence required to produce a functional phytoene dehydrogenase-4H enzyme.

Because of the introduction of a Nco I site at the initiation methionine of the gene, the nucleotide sequence was slightly changed:

Original sequence:

(SEQ ID NO:34)
5' TAA AGG ATG AAA AAA ACC GTT GTG ATT GGC 3'
MET Lys Lys Thr Val Val Ile Gly
(SEQ ID NO:35)

New Genetically Engineered Sequence:

(SEQ ID NO:36)
5' TAA ACC ATG GAA AAA ACC GTT GTG ATT GGC 3'
MET Glu Lys Thr Val Val Ile Gly
(SEQ ID NO:37)

The sequence at the 3' end of the gene was not changed as a result of the PCR reaction.

g. Phytoene Dehydrogenase-4H Assay

The assay for phytoene dehydrogenase-4H was developed using two R. sphaeroides mutants, I-3 and E-7. I-3, a mutant strain that has a mutation in the gene for phytoene dehydrogenase-3H, was provided by Dr. Samuel Kaplan, University of Texas Medical Center, Houston, Texas. This mutant, which accumulates phytoene, was used as a source of the substrate for phytoene dehydrogenase-3H and phytoene dehydrogenase-4H.

R. sphaeroides E-7 is a strain that cannot make any carotenoids, and was developed at the Amoco Research Center, Naperville, Illinois. This mutant, which has an intact gene for a different, but similar phytoene dehydrogenase-3H, provided a source of the similar enzyme to determine the proper assay conditions.

The membrane fraction from the Rhodobacter I-3 mutant was isolated by growing I-3 cells until mid to late log phase, pelleting and lysing the harvested cells in 100 mM Tris Buffer, pH 8.0, by vortexing with 150 micron acid-washed glass beads. The cell homogenate was then used as the source of phytoene.

Although the R. sphaeroides E-7 phytoene dehydrogenase-3H transforms phytoene to either phytofluene or neurosporene but not to lycopene, as in Erwinia herbicola, the assay conditions delineated for the Rhodobacter enzyme were also efficacious for the Erwinia herbicola phytoene dehydrogenase-4H. These conditions were used to detect phytoene dehydrogenase-4H activity in both E. coli and S. cerevisiae harboring the Erwinia herbicola structural gene for phytoene dehydrogenase-4H, as is discussed below.

-115-

To isolate the phytoene dehydrogenase-4H from either bacteria or yeast harboring the Erwinia herbicola gene, cells were grown until mid-late log phase and harvested by pelleting. The cell pellet was either frozen for later use or used immediately. A frozen or fresh cell pellet was resuspended in one volume of 100 mM Tris Buffer, pH 8.0, and lysed by vortexing as described above for Rhodobacter (150 micron beads were used to lyse bacteria and 450 micron beads were used to lyse yeast). This cell lysate provided a source of phytoene dehydrogenase-4H for testing.

An aliquot of the Erwinia herbicola phytoene dehydrogenase-4H-containing lysate was admixed with an aliquot of the Rhodobacter I-3 cell lysate described above in a buffer containing 100 mM Tris, pH 8.0, 10 mM ATP, 2.5 mM NADP, 4 mM DTT, 4 mM MgCl₂, 6 mM MnCl₂ in a total volume of 1-2 ml. The reaction mixture was incubated at 30°C in the dark for 2-8 hours, and the contents were extracted first with hexane and then with chloroform. The organic layers were pooled, dried, and analyzed by HPLC on a C-18 analytical column (4.6 X 250 mm) developed with a linear gradient, starting with 30 percent isopropyl alcohol and 70 percent acetonitrile:water (9:1) and ending with 55 percent isopropyl alcohol and 45 percent acetonitrile:water (9:1), in 30 minutes at a flow rate of 1 ml/minute. Lycopene, which eluted at about 16.2 minutes, was quantitated from a predetermined standard curve.

-116-

Example 9. Lycopene Production in *E. coli*

a. Method One - Plasmid(s) containing the engineered genes for GGPP synthase, phytoene synthase and phytoene dehydrogenase

5 Active GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H enzymes can convert ubiquitous cellular precursors into lycopene. Lycopene was produced in *E. coli* when plasmids containing the three genes for the above enzymes were
10 introduced into the bacterial host cells. One combination producing lycopene utilized host cells transformed with the plasmids pARC275 and pARC496A.

The plasmid pARC275 was constructed in the following manner. First, the plasmid pARC376-Pst 110
15 was made by deleting the about 2999 bp Pst I segment (between positions 7792 and 10791, Figure 5) from plasmid pARC376 as described before. Second, the Eco RI (3370) to Hind III (13463 Figure 5) segment from plasmid pARC376-Pst 110 was excised and cloned into
20 the Eco RI to Hind III sites of plasmid pSOC925 to produce plasmid pARC275.

The plasmid pSOC925 is about a 9 kilobase plasmid whose restriction map is illustrated in Figure 12. This plasmid contains the kanamycin and
25 chloramphenicol (CAT) resistance genes and the R1162 origin of replication. The chloramphenicol resistance gene can be excised from the plasmid by digestion with Eco RI and Hind III (Figure 12).

The fragment (Eco RI to Hind III of plasmid pARC376-Pst 110) containing the relevant portion of
30 the Erwinia herbicola carotenoid genes was isolated. Plasmid pSOC925 was digested with Eco RI and Hind III, excising the CAT gene. About 100 ng of the larger portion of digested plasmid pSOC925 was
35 admixed with about 200 ng of the Eco RI to Hind III

-117-

fragment from pARC376-Pst 110 in a total volume of 20 μ l to which 2 μ l of Ligation Buffer and 1 Unit of T4 Ligase were added. The ligation mixture was incubated at 4°C for about 15 hours and then transformed into E. coli HB101 cells. Transformants were grown in Luria-Broth supplemented with 25 μ g/ml of kanamycin. DNA was isolated from prospective clones and those clones containing the desired DNA insert were identified by restriction analysis. The resultant pARC275 plasmid confers the ability to produce phytoene on E. coli.

Transformation of E. coli host cells with plasmids pARC275 and pARC496A produced red colonies of the transformed host cells, as is discussed in Example 8.

b. Method Two - Plasmid with a defective gene for lycopene cyclase

Following production of lycopene, the next step in the Erwinia herbicola biosynthetic pathway is the transformation of lycopene to beta-carotene by lycopene cyclase. When the gene encoding lycopene cyclase is inhibited, mutated, or in some other manner made non-functional, the enzyme lycopene cyclase, which transforms lycopene to beta-carotene, does not function. Lycopene accumulates when this occurs.

The plasmid pARC376-Ava 102, a derivative of plasmid pARC376 in which the gene for lycopene cyclase has been deleted, was constructed by partially digesting plasmid pARC376 with Ava I to remove two adjacent, relatively short Ava I-Ava I fragments and relegating the cut ends of the remaining, relatively large fragment. The two relatively small Ava I-Ava I fragments included the

-118-

about 1633 bp Ava I fragment (10453-8842 Figure 5) and the about 611 bp Ava I-Ava I fragment from (8842-8231 Figure 5). In total, about 2222 bp of DNA were deleted from the plasmid pARC376.

5 The resulting plasmid pARC376-Ava 102 was transformed into E. coli HB101, and the transformants were grown on Luria-Broth with 100 µg/ml of ampicillin. Normally, E. coli cells that contain the entire plasmid pARC376 are yellow due to the
10 production of zeaxanthin and zeaxanthin derivatives. Following transformation, some of the clones were now red in color.

 Plasmid DNA was isolated from one of these red E. coli clones and subjected to restriction
15 analysis, which revealed that the two Ava I-Ava I fragments had been deleted from the original pARC376 plasmid. This deletion of the Ava I fragments from plasmid pARC376 impaired the gene for lycopene cyclase.

20 Under this circumstance, the three genes for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H on plasmid pARC376-Ava 102 functioned properly and produced lycopene. Because
25 the gene for lycopene cyclase did not function properly, the transformed E. coli host cells accumulated lycopene.

Example 10. Lycopene Production in S. cerevisiae

 Normal yeast cells do not produce lycopene.
30 Genes sufficient to make lycopene in S. cerevisiae include those for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H. The plasmid pARC145G (Example 5) has the genes for GGPP synthase and
35 phytoene synthase on both sides and adjacent to the GAL 10 and GAL 1 divergent promoter region. Both of

-119-

these genes are expressed in S. cerevisiae using these two promoters.

5 The gene for phytoene dehydrogenase-4H is located on the plasmid pARC146D described hereinafter. These two plasmids were transformed into S. cerevisiae, strain YPH499.

10 The yeast strain YPH499 contains a non-functional TRP 1 gene and a non-functional URA 3 gene (as discussed in Example 5). Plasmid pARC145G contains a functioning TRP 1 gene as well as the genes for GGPP synthase and phytoene synthase. Plasmid pARC146D contains a functioning URA 3 gene as well as the gene for phytoene dehydrogenase-4H. After both plasmids were introduced, the yeast cells
15 were grown on Medium 4 (Example 5) with galactose to induce the expression of the three carotenoid genes.

20 The cells were grown to stationary phase, collected, extracted, and analyzed by HPLC according to the protocols described before. Yeast cells with the three carotenoid structural genes produced lycopene at about 0.01 percent dry weight.

a. Construction of Plasmid pARC146

25 The plasmid pARC146 is a S. cerevisiae vector constructed to direct the expression of the phytoene dehydrogenase-4H gene in yeast.

30 The construction of plasmid pARC145B (Figure 9) was outlined before in Example 5 for production of phytoene. Two modifications were made to plasmid pARC145B in order to construct plasmid pARC146.

The first modification was the introduction of the PGK terminator at the Sph I site of plasmid pARC145B, downstream from the GAL 1 promoter. A polycloning site, into which a structural gene could

-120-

be cloned, is present between the GAL 1 promoter and the PGK terminator.

Thus, an about 500 bp Eco RI-Hind III fragment containing the S. cerevisiae PGK terminator was excised from plasmid pARC117 (Example 5). This is substantially the same PGK terminator fragment discussed in Example 5 and shown in Figure 7 for plasmid pARC135. The Eco RI and Hind III ends of this fragment were blunted by treatment with the Klenow fragment of DNA Polymerase. Synthetic double-stranded sequences each containing a potential Sph I cleavage site (BRL) were then ligated to both ends of the PGK terminator fragment, and that fragment was digested with Sph I, producing sticky ends. Plasmid pARC145B was digested with Sph I, and the Sph I-linkered PGK terminator was ligated to form the resulting plasmid pARC145C.

The second modification was to replace the yeast TRP 1 gene with the yeast URA 3 gene. This enabled transfer of the plasmid into yeast cells that had a mutation in the URA 3 gene on the yeast chromosome. Here, the plasmid pARC145C was digested with restriction enzymes Msc I and Eco RV, and a 737 bp fragment containing the TRP 1 gene was deleted.

Synthetic double-stranded sequences containing a potential Xho I cleavage site (BRL) were ligated to the Msc I and Eco RV blunt ends (there are no other Xho I sites in plasmid pARC145). The resulting DNA fragment was digested with Xho I to produce a DNA having Xho I sticky ends.

Meanwhile, an about 1000 bp Hind III fragment, including the entire URA 3 gene, was excised from the plasmid YEp24 (ATCC 37051). The ends of this fragment were blunted with the Klenow fragment of DNA Polymerase. Synthetic double-

-121-

stranded sequences, each containing a potential Xho I cleavage site were ligated to the blunt ends. This fragment was then digested with Xho I, producing sticky ends.

5 This URA 3 gene fragment was then ligated into the Xho I-digested pARC145C plasmid (from which the TRP 1 gene had been deleted). The final plasmid was named pARC146 and is similar to plasmid pARC145C except that plasmid pARC146 contains a URA 3
10 selectable maker instead of a TRP 1 gene.

 Unexpectedly, plasmid pARC146 did not contain two Xho I sites. The Xho I site expected at the location of the Eco RV site of the original vector, denoted as (Xho I) in Figure 13, could not be
15 digested. However, the apparent loss of the site did not effect the utility of plasmid pARC146 as a URA 3 selectable vector and also did not effect the utility of plasmid pARC146 as an expression vector.

 The relevant features of this new plasmid
20 construct are i) the presence of the divergent GAL 1 and GAL 10 promoters, ii) the PGK terminator at the 3' end of the GAL 1 promoter, iii) the 2 micron STB terminator (2 MIC STB TERM) at the 3' end of the GAL 10 promoter, iv) the URA 3 gene that is the
25 selectable marker for transferring the plasmid into S. cerevisiae, and v) the 2 micron origin of replication that permits the maintenance of the plasmid in yeast. This plasmid also contains the pMB1 origin of replication for maintenance in E. coli
30 and the ampicillin resistance gene for selection in E. coli. A restriction map of the plasmid pARC146 is shown in Figure 13.

-122-

b. Construction of Plasmid pARC496B

Plasmid pARC496B was constructed to introduce a Sal I site immediately upstream from the initiation methionine of the phytoene dehydrogenase-4H structural gene and a Sal I site at the 3' end of the gene to enable the gene for phytoene dehydrogenase-4H to be moved as a Sal I-Sal I fragment. This version of the gene was used as the structural gene for phytoene dehydrogenase-4H in constructing the plasmid pARC146D (described below) that was transformed into S. cerevisiae in combination with transformation with plasmid pARC145G to cause the production of lycopene in the transformed yeast. The plasmid pARC496B was constructed using the PCR protocol described before (plasmid pARC496A) to introduce Sal I sites at the 5' and 3' end of the gene.

i. Template DNA for the PCR

The plasmid pARC271D (Example 8) was digested with Sal I and Xmn I and an about 3035 bp fragment (9340-6305, Figure 5) was isolated after separation on agarose gel electrophoresis. This fragment was used as the template for PCR.

ii. Probes for the PCR

Two oligonucleotide probes were used to introduce Sal I sites at the 5' and the 3' ends of the gene for phytoene dehydrogenase-4H. At the 5' end of the gene, the newly introduced Sal I site was immediately upstream from the initiation methionine. At the 3' end of the gene, the newly introduced Sal I site was immediately upstream from the Nco I site at 6342.

-123-

The original sequence of the 5' end was:

5' G AGA TAA AGG ATG AAA AAA ACC GTT GTG AT 3'
MET ... (SEQ ID NO:38)

5

The oligonucleotide probe for the 5' end was:

5' G AGG Sal I TCG ACG ATG AAA AAA ACC GTT GTG AT 3'
MET ... (SEQ ID NO:39)

10

The second strand oligonucleotide probe for the 3' end of the gene was:

5' AT GGT CGA CGT GGC GTG GTC AAG CAG CGG 3' (SEQ ID NO:40)

15

The polymerase chain reaction was carried out as described before. After completion, the reaction mixture was extracted twice with ether and the DNA was precipitated with ethanol.

20

iii. Cloning of the PCR produced DNA Fragment

The DNA accumulated from the PCR was digested with Sal I, producing an about 1508 bp fragment (from the "T" of the TCGAC overhang at the 5' end of the gene to the "G" of the Sal I site at the 3' end of the gene). Five μ g of the plasmid pARC306A (Figure 6) was digested with Sal I. About 100 ng of the Sal I-digested plasmid pARC306A and about 200 ng of the Sal I-Sal I fragment of the phytoene dehydrogenase-4H structural gene prepared by PCR were admixed with 2 μ l of Ligation Buffer (IBI) and 1 Unit of T4 Ligase in a total volume of 20 μ l. The ligation reaction mixture was incubated at 4°C for about 15 hours.

25

30

The resulting plasmid was transformed into *E. coli* HB101, and the transformants were selected by growth in Luria-Broth supplemented with 100 μ g/ml of ampicillin. DNA from prospective clones was isolated and the identity of clones containing the phytoene

35

-124-

dehydrogenase-4H gene was confirmed by restriction enzyme analysis.

The resultant plasmid was named pARC496B. The about 1508 bp Sal I-Sal I fragment (also referred to as a Sal I fragment), another particularly preferred DNA segment herein, was cloned from plasmid pARC496B into the yeast vector pARC146, to generate the plasmid pARC146D as described hereinafter.

iv. Sequence of the Phytoene Dehydrogenase-4H Gene Fragment of Plasmid pARC496B

The introduction of the Sal I sites at the 5' and 3' ends of the gene for phytoene dehydrogenase-4H changed the nucleotide sequence of the native DNA fragment slightly.

Original sequence at the 5' end of the gene:

5' GAG ATA AAG G ATG AAA AAA ACC GTT GTG AT 3' (SEQ ID NO:41)
MET Lys Lys Thr Val Val ... (SEQ ID NO:42)

Sequence of the genetically engineered versions of the gene at the 5' end:

Nco I (SEQ ID NO:43)
5' CC ATG GAA AAA ACC GTT GTG AT 3'
MET Glu Lys Thr Val Val (SEQ ID NO:44)

Sal I (SEQ ID NO:45)
5' GAG GTC GAC G ATG AAA AAA ACC GTT GTG AT 3'
MET Lys Lys Thr Val Val ... (SEQ ID NO:46)

Original sequence at the 3' end of the gene:

(SEQ ID NO:47)
Nco I (6395)
5' CC GCT GCT TGA CCA CGC CAC GCA GAC CAT GG 3'

-125-

After the introduction of the Sal I site from the PCR reaction the new sequence became:

5' CC GCT GCT TGA CCA CGC CAC Sal I Nco I (6395)
GTC GAC CAT GG 3'
(SEQ ID NO:48)

c. Construction of the plasmid pARC146D

An about 1508 bp Sal I fragment described above containing the structural gene for phytoene dehydrogenase-4H was excised from plasmid pARC496B and was ligated into the Sal I site of the pARC146 plasmid described before. The result was the plasmid pARC146D construct, placing the gene for phytoene dehydrogenase-4H between and adjacent to the GAL 1 promoter and the PGK terminator. A restriction map of the pARC146D plasmid is illustrated in Figure 14, in which the location of the phytoene dehydrogenase-4H gene is shown as "PDH".

Example 11. Expression of *Erwinia herbicola* Phytoene Dehydrogenase-4H Gene in *Rhodobacter sphaeroides*

This Example describes the construction of a plasmid, pATC228, that was transformed into a mutant strain of *R. sphaeroides*, causing the expression of *Erwinia herbicola* phytoene dehydrogenase-4H in that organism. Plasmid vector pATC228 was made by combining the plasmid pATC1619, which contains a genetically engineered phytoene dehydrogenase-4H structural gene, with plasmid pSOC244, which is capable of transforming and being maintained in both *E. coli* and *R. sphaeroides*. The following is a description of the multistep construction of plasmid pATC228.

-126-

a. Construction of Plasmid pATC1619

The plasmid pATC1619 contains a genetically engineered version of the phytoene dehydrogenase-4H gene cloned adjacent to the TAC promoter of pDR540 (Pharmacia). The gene for phytoene dehydrogenase-4H is expressed in E. coli and photosynthetic bacteria using the TAC promoter. Plasmid pATC1619 was constructed in a multistep procedure requiring several intermediate plasmids as outlined below.

i. Plasmid pARCBgIII401

The plasmid pARCBgIII401 was constructed by cloning the about 5513 bp Bgl II fragment from plasmid pARC376 (from position 6836 to position 12349 in Figure 5) into the Bam HI site of plasmid pARC306A (Figure 6).

ii. Plasmid pATC1403

An about 1548 bp Pst I to Sal I fragment from plasmid pARCBgIII401 (original coordinates in Figure 5 were 7792 and 9340, respectively) was cloned into the Pst I and Sal I sites of plasmid M13mp19 (BRL) to generate plasmid pARC1403. Plasmid pATC1403 contains a beginning portion of the phytoene dehydrogenase-4H gene.

iii. Plasmid pATC1404

A Sph I site was introduced at the initiation MET codon of the phytoene dehydrogenase-4H gene in plasmid pATC1403, using the in vitro mutagenesis protocol described in Current Protocols in Molecular Biology, Ausabel et al. eds., John Wiley & Sons, New York (1987), pp. 8.1.1-8.1.6 (see Example 2). The oligonucleotide probe used as the primer was:

-127-

5' G ACG AGA TAA AGC ATG CAA AAA ACC GTT GT 3' (SEQ ID NO:49)
MET Gln Lys Thr Val ... (SEQ ID NO:50)

5

The sequence in the native phytoene dehydrogenase-4H gene was:

5' G ACG AGA TAA AGG ATG AAA AAA ACC GTT GT 3' (SEQ ID NO:51)
MET Lys Lys Thr Val ... (SEQ ID NO:52)

10

As a result of the introduction of the Sph I site, the second amino acid of the phytoene dehydrogenase-4H enzyme was changed from Lys to Gln. Thus, the new sequence became:

15

5' G ACG AGA TAA AGC ATG CAA AAA ACC GTT GT 3' (SEQ ID NO:53)
MET Gln Lys Thr Val (SEQ ID NO:54)

20

This plasmid, with the Sph I site at the initiation methionine codon of the phytoene dehydrogenase-4H structural gene, was named pATC1404.

25

iv. Plasmid pATC816

The plasmid, pARC306A (Figure 6) was digested with Pst I and Sma I. The plasmid pARC376 (Figure 5) was digested with Pst I and Bal I. An about 1451 bp Pst I (7792) to Bal I (6341) fragment was isolated from an agarose gel. Both, Bal I and Sma I digestions leave a blunt end. The approximately 1451 bp Pst I-Bal I fragment from plasmid pARC376 was cloned into the Pst I and Sma I digested plasmid pARC306A to form plasmid pATC816.

30

35

Plasmid pARC306A contains an Eco RI site about 30 bp downstream from the Sma I site. The Eco RI site originally present in plasmid pARC306A is maintained in plasmid pATC816.

40

v. Plasmid pATC1605

As previously stated, the plasmid pATC1404 contains only the beginning portion of the gene encoding phytoene dehydrogenase-4H. To fuse this
5 portion with the remainder of the phytoene dehydrogenase-4H gene, an about 1052 bp Sma I to Pst I fragment from plasmid pATC1404 (original position 8844 to 7792 of pARC376 in Figure 5) was excised and cloned into plasmid pATC816 (which
10 contains the 3' portion of the phytoene dehydrogenase-4H gene) as follows.

Plasmid pATC816 was digested with Ssp I and Pst I (both sites are unique in the pATC816 plasmid). Digestion with Ssp I left a blunt end. The about
15 1052 bp Sma I (blunt end) to Pst I fragment from plasmid pATC1404 was cloned into the digested plasmid pATC816, resulting in plasmid pATC1605.

This cloning procedure (Sma I to Pst I fragment of plasmid pATC1404 into plasmid pATC816) resulted in the complete sequence of the phytoene
20 dehydrogenase-4H gene becoming contiguous in plasmid pATC1605. There is a superfluous DNA segment immediately upstream from the initiation codon of the phytoene dehydrogenase-4H gene.

In addition, the newly created Sph I site of plasmid pATC1404 containing the codon for the initial
25 Met residue of the enzyme became a part of the phytoene dehydrogenase-4H structural gene. The originally present Nco I site shown near the 3' end of the sequence of Figure 11-4 is also present in
30 this construct as is the Eco RI site downstream therefrom that was introduced from plasmid pARC306A. The Sph I-Eco RI fragment of plasmid pATC1605 that contains the structural gene for phytoene
35 dehydrogenase-4H contains about 1550 bp.

-129-

vi. Plasmid pATC1607

Plasmid pATC1605 was digested with Sph I and Eco RI enzymes. The resultant fragment of about 1550 bp was cloned into the plasmid pUC19 (Pharmacia), which had been digested with Sph I and Eco RI enzymes, resulting in the plasmid, pATC1607.

vii. Plasmid pATC1619

Upstream and adjacent to the Sph I site on plasmid pATC1607 is a Hind III site that originates from the polylinker region of plasmid pUC19. The structural gene for phytoene dehydrogenase-4H was excised from plasmid pATC1607 by digesting with Hind III and Eco RI. The ends of the resultant fragment, also of about 1550 bp, were blunted by treating with the Klenow fragment of E. coli DNA Polymerase.

The plasmid, pDR540 (Pharmacia), which contains the TAC promoter for gene expression in some bacteria, including E. coli and R. sphaeroides, and a unique Bam HI site downstream of the TAC promoter, was digested with Bam HI, and the ends were blunted as above. The blunt ended DNA fragment from plasmid pATC1607 (above) was cloned into plasmid pDR540, resulting in the plasmid pATC1619, which contained the bacterial TAC promoter adjacent to the structural gene for phytoene dehydrogenase-4H. Plasmid pATC1619 also contains a unique Hind III site.

b. Construction of Plasmid pATC228

Plasmid pSOC244 is a plasmid that contains i) the R1162 origin of replication, ii) the chloramphenicol acetyltransferase gene that confers resistance to chloramphenicol adjacent to the TAC promoter, and iii) a unique Hind III site. This plasmid can transform and be maintained in both

-130-

E. coli and R. sphaeroides. The construction of plasmid pSOC244 is discussed below.

i. Plasmid pSOC200

5 Plasmid pQR176a was obtained from Dr. J.A. Shapiro of the University of Chicago, Chicago, IL, and is described in Meyer et al., J. Bacteriol., 152:140 (1982). This plasmid contains the R1162 origin of replication and the transposon Tn5, which
10 confers resistance to kanamycin. This plasmid contains about 14.5 kilobases and contains several Hind II restriction sites.

Digestion of plasmid pQR176a with Hind II, followed by religation of appropriate fragments
15 provided plasmid pSOC200, which contained about 8.5 kilobases. This plasmid retained the R1162 origin of replication and the kanamycin resistance gene from Tn5.

20 ii. Plasmid pSOC244

Plasmid pSOC200 was digested with Hind III and Sma I endonucleases to remove the kanamycin resistance gene. Plasmid pSOC925 was similarly
25 digested to provide an approximately 1000 bp fragment containing the chloramphenicol acetyltransferase (CAT) structural gene with the adjacent TAC promoter. That approximately 1000 bp fragment was then cloned into the Hind III- and Sma I-digested plasmid pSOC200 fragment to provide plasmid pSOC244.

30

iii. Plasmid pATC228

Both plasmids, pATC1619 and pSOC244, were digested with Hind III. The two plasmids were
35 ligated together and selected in E. coli grown in medium containing ampicillin (using the ampicillin

-131-

resistance gene from the pATC1619 plasmid) and chloramphenicol (using the chloramphenicol resistance gene from the pSOC244 plasmid). The resultant plasmid was pATC228, which contains the structural gene for phytoene dehydrogenase-4H and can transform and be maintained in R. sphaeroides. This structural gene can be excised from plasmid pATC228 as an approximately 1506 bp Sph I-Nco I restriction fragment. Plasmid pATC228 is shown schematically in Figure 16.

c. Expression of the Erwinia Herbicola Phytoene Dehydrogenase-4H Gene in a R. sphaeroides I-3 Mutant

The R. sphaeroides I-3 mutant (utilized in Example 8g), possesses an impaired native crtI gene for phytoene dehydrogenase-3H, and thus accumulates phytoene. Cells from R. sphaeroides I-3 were transformed as hosts with plasmid pATC228. The transformants were selected in the presence of chloramphenicol. The mutant cells that were previously colorless, were colored red after transformation. The red pigment produced by these cells had physicochemical characteristics that were consistent with the properties of the carotenoid spirilloxanthin.

The pigment produced by the plasmid pATC228-transformed R. sphaeroides I-3 mutant host cells was compared to authentic spirilloxanthin extracted from R. rubrum (ATCC 25903) cells grown in culture. The two pigments had the same UV-Vis spectra and the same HPLC profiles. Spirilloxanthin from R. rubrum is derived from lycopene through a series of catalytic steps that include two dehydrogenations, hydration, and then methylation. The Photosynthetic Bacteria,

-132-

Roderick et al. eds., Plenum Press, New York, pages 729-750 (1978).

5 R. sphaeroides normally transforms phytoene to neurosporene, but not to lycopene, as is the case in Erwinia herbicola. It is believed, therefore, that in the production of the spirilloxanthin-like pigment in the transformed R. sphaeroides, the Erwinia herbicola phytoene dehydrogenase-4H catalyzed
10 desaturation of accumulated phytoene to produce lycopene. The produced lycopene was thereafter further metabolized by native enzymes present in the R. sphaeroides mutant to form spirilloxanthin-like carotenoid.

15 **Example 12. Lycopene Production in Pichia pastoris**

The above-described method is also extendable to other yeasts. One yeast system that serves as an example is the methylotrophic yeast, Pichia pastoris.

20 To produce lycopene in P. pastoris, structural genes for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H are placed under the control of regulatory sequences that direct expression of structural genes in Pichia. The resultant expression-competent forms of those genes
25 are introduced into Pichia cells.

For example, the transformation and expression system described by Cregg et al., Biotechnology 5:479-485 (1987); Molecular and Cellular Biology 12:3376-3385 (1987) can be used. A
30 structural gene for GGPP synthase such as that from plasmid pARC489D is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. Similarly, structural genes for
35 phytoene synthase and phytoene dehydrogenase-4H such

-133-

as that from plasmids pARC140N and pARC146D are placed between AOX1 promoters and terminators. All three of these genes and their flanking regulatory regions are then introduced into a plasmid that carries both the P. pastoris HIS4 gene and a P. pastoris ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within P. pastoris cells [Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987)].

The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in E. coli cells. The final resultant plasmid carrying GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H genes, as well as the various additional elements described above, is illustratively transformed into a his4 mutant of P. pastoris, i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987), to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the enzymes GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H and the production of lycopene in P. pastoris.

The three genes for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987).

-134-

Example 13. Lycopene Production in *A. nidulans*

The genes encoding GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H as discussed before can be used to synthesize and accumulate lycopene in fungi such as *Aspergillus nidulans*. Genes are transferred to *Aspergillus* by integration.

For example, the structural gene for GGPP synthase is introduced into the *E. coli* plasmid pBR322. The promoter from a cloned *Aspergillus* gene such as *argB* [Upshall et al., *Mol. Gen. Genet.* 204:349-354 (1986)] is placed into the plasmid adjacent to the GGPP synthase structural gene. Thus, the GGPP synthase gene is now under the control of the *Aspergillus argB* promoter.

Next, the entire cloned *amds* gene [Corrick et al., *Gene* 53:63-71 (1987)] is introduced into the plasmid. The presence of the *amds* gene permits acetamide to be used as a sole carbon or nitrogen source, thus providing a means for selecting those *Aspergillus* cells that have become stably transformed with the *amds*-containing plasmid.

Thus, the plasmid so prepared contains the *Aspergillus argB* promoter fused to the GGPP synthase gene and the *amds* gene present for selection of *Aspergillus* transformants. *Aspergillus* is then transformed with this plasmid according to the method of Ballance et al., *Biochem. Biophys. Res. Commun.* 112:284-289 (1983).

The GGPP synthase, phytoene synthase and phytoene dehydrogenase-4H structural genes are each similarly introduced into the *E. coli* plasmid pBR322. Promoters for the cloned *Aspergillus argB* gene [Upshall et al., *Mol. Gen. Genet.* 204:349-354 (1986)] are placed immediately adjacent to the phytoene synthase and phytoene dehydrogenase-4H structural

-135-

genes. Thus, these structural genes are controlled by the Aspergillus argB promoters.

The entire, cloned Aspergillus trpC gene [Hamer and Timberlake, Mol. Cell. Biol., 7:2352-2359 (1987)] is introduced into the plasmid. The trpC gene permits selection of the integrated plasmid by virtue of permitting transformed trpC mutant Aspergillus cells to now grow in the absence of tryptophan. The Aspergillus strain, already transformed with the plasmid containing the GGPP synthase gene, is now capable of synthesizing lycopene.

Example 14. Phytoene Dehydrogenase-4H in Higher Plants

Higher plants have the genes encoding the enzymes required for lycopene production and so inherently have the ability to produce lycopene. Lycopene normally is not accumulated, however, because lycopene so produced in most plants is further converted to other products. Even in the case of ripe tomato fruits, the level of lycopene accumulated is only about 0.01 percent dry weight. The carotenoid-specific genes from Erwinia herbicola can be used to express phytoene dehydrogenase-4H for use by the plant as well as to improve accumulation of lycopene in plants. Two useful approaches are described below.

a. Transport to the Chloroplast

In the first approach, the gene for phytoene dehydrogenase-4H was modified to introduce the restriction site Sph I at the initiation methionine codon, as discussed before. An about 177 bp DNA fragment that encodes for the transit (signal)

-136-

peptide of the tobacco gene for ribulose bis-phosphate carboxylase-oxygenase containing a Nco I site at the 5' end and a Sph I site at the 3' end, was ligated to the Sph I site of the structural phytoene dehydrogenase-4H gene. This modified gene was inserted into the plasmid pCaMVCN (Pharmacia, Piscataway, N.J.) replacing the CAT gene. The resultant plasmid contained a gene for phytoene dehydrogenase-4H with the transit peptide sequence placed between and adjacent to both the CaMV 35S plant promoter and the NOS polyadenylation sequence at the 3' end.

This phytoene dehydrogenase-4H gene construct was inserted into the plasmid pGA482 (Pharmacia) in a convenient restriction site within the multiple cloning linker region to form plasmid pATC1616. The relevant features of plasmid pGA482 include (i) an origin of replication that permits maintenance of the plasmid in Agrobacterium tumefaciens, (ii) the left and right border sequences from the T-DNA region that direct the integration of the DNA segment between the borders into the plant genome, and (iii) the NOS promoter adjacent to the kanamycin resistance gene that permits plant cells to survive in the presence of kanamycin.

This phytoene dehydrogenase-4H gene construct was transformed into Agrobacterium tumefaciens LBA4404 (Clontech, Inc.) according to standard protocols. Agrobacterium cells containing the plasmid with the phytoene dehydrogenase-4H gene construct were transferred by infection of tobacco leaf discs using the method of Horsch et al., Science, 227:1229-1231 (1985). During the infection process, the entire DNA segment between the left and right borders of the plasmid pGA482 plasmid is

-137-

transfected into the plant cells. Transfected plant cells are selected for kanamycin resistance.

Transgenic tobacco plants were grown in the presence of the herbicide norflurazon (Sandoz).
5 Control plants that had been transformed with the control plasmid pGA482 and that did not contain Erwinia herbicola phytoene dehydrogenase-4H structural gene bleached when grown in the presence of 0.2 $\mu\text{g/ml}$ in the growth medium. Transgenic plants
10 containing the Erwinia herbicola phytoene dehydrogenase-4H structural gene grew normally in the presence of 0.8 $\mu\text{g/ml}$ of Norflurazon. Thus, the introduction of the Erwinia herbicola phytoene dehydrogenase-4H structural gene caused the
15 expression of Erwinia herbicola phytoene dehydrogenase-4H, and plants to become resistant to a herbicidal amount of norflurazon.

The specific DNA segments, recombinant molecules and techniques utilized in the preparation
20 of the above norflurazon-resistant tobacco plants are discussed below.

1. Transit Peptide

The sequence of the transit peptide DNA
25 is basically that of Mazur et al., Nucl. Acids Res., 13:2343-2386 (1985) for the ribulose bis-phosphate carboxylase-oxygenase signal peptide of Nicotiana tabacum. Two changes were made to the disclosed 177 bp sequence.

30 In the first change, two cytidine residues were added at the 5' end to create a Nco I restriction site. The second change introduced an Nar I site that cleaves between bases at positive 73 and 74. This change was a G for T replacement at
35 position 69 and a G for A replacement at position 72,

-138-

both of which changes left the encoded amino acid residue sequence unchanged. The final two residues at the 3' end were deleted to provide the natural Sph I restriction site sticky end.

5 The synthetic transit peptide-encoding DNA also therefore contained 177 bp. The complete double stranded sequence, showing the 5' Nco I and 3' Sph I sticky ends, is illustrated in Figure 17.

10 The DNA encoding the transit peptide was synthesized synthetically from eight fragments that were annealed together in pairs by heating at 90°C for five minutes and then slowly cooling to room temperature. Fifty picomoles of each fragment were utilized.

15 Those eight fragments were:

1. 5' CAT GGC TTC CTC AGT TCT TTC CTC TGC AGC AGT
TGC C 3' (SEQ ID NO:55)

20 2. 5' GGG TGG CAA CTG CTG CAG AGG AAA GAA CTG AGG
AAG C 3' (SEQ ID NO:56)

3. 5' ACC CGC AGC AAT GTT GCT CAA GCT AAC ATG
GTG G 3' (SEQ ID NO:57)

25 4. 5' CGC CAC CAT GTT AGC TTG AGC AAC ATT GCT GC 3'
(SEQ ID NO:58)

5. 5' CGC CTT TCA CTG GCC TTA AGT CAG CTG CCT CAT
TCC CTG TTT CAA GGA AG 3' (SEQ ID NO:59)

30 6. 5' TTT GCT TCC TTG AAA CAG GGA ATG AGG CAG CGA
ATG AGG CAG CTG ACT TAA GGC CAG TCA AAG G 3'
(SEQ ID NO:60)

35

-139-

7. 5' CAA AAC CTT GAC ATC ACT TCC ATT GCC AGC AAC
GGC GGA AGA GTG CAA TGC ATG 3'
(SEQ ID NO:61)

5 8. 5' CAT TGC ACT CTT CCG CCG TTG CTG GCA ATG GAA
GTG ATG TCA AGG T 3' (SEQ ID NO:62)

The pairs utilized for annealing were 1 and
2, 3 and 4, 5 and 6, and 7 and 8 to form sticky ended
annealed pairs 1-2, 3-4, 5-6 and 7-8 that are shown
below.

1-2

(SEQ ID NO:63)
5' CATGGCTTCCTCAGTTCTTTCCTCTGCAGCAGTTGCC 3'
15 3' CGAAGGAGTCAAGAAAGGAGACGTCGTCAACGGTGGG 5'
(SEQ ID NO:64)

3-4

(SEQ ID NO:65)
5' ACCCGCAGCAATGTTGCTCAAGCTAACATGGTGG 3'
20 3' CGTCGTTACAACGAGTTCGATTGTACCACCGC 5'
(SEQ ID NO:66)

5-6

5' CGCCTTTCCTGAGCTTAAAGTCAGCTGCCTCATTCCCTGTTTCA
25 3' GGAAAGTGACCGGAATTCAGTCGACGGAGTAAGGGACAAAGT

AGGAAG 3' (SEQ ID NO:67)
TCCTTCGTTT 5' (SEQ ID NO:68)

30 7-8

5' CAAAACCTTGACATCACTTCCATTGCCAGCAACGGCGGAAGAGT
3' TGGAAGTGTAGTGAAGGTAACGGTCGTTGCCGCTTCTCA

35 GCAATGCATG 3' (SEQ ID NO:69)
CGTTAC 5' (SEQ ID NO:70)

Fragment 1-2 was ligated with fragment 3-4 to
form fragment 1-4 whose sequence is shown below.

5' CATGGCTTCCTCAGTTCTTTCCTCTGCAGCAGTTGCCACCGCAGCAA
3' CGAAGGAGTCAAGAAAGGAGACGTCGTCAACGGTGGGCGTCGTT
45 TGTTGCTCAAGCTAACATGGTGG 3' (SEQ ID NO:71)
ACAACGAGTTCGATTGTACCACCGC 5' (SEQ ID NO:72)

-140-

Fragment 5-6 was ligated with fragment 7-8 to form fragment 5-8 whose sequence is shown below.

5' CGCCTTTCACTGGCCTTAAGTCAGCTGCCTCATTCCCTGTTTCAAGGA
3' GGAAAGTGACCGGAATTCAGTCGACGGAGTAAGGGACAAAGTTCCT

AGCAAAACCTTGACATCACTTCCATTGCCAGCAACGGCGGAAG
TCGTTTTGGAAGTGTAGTGAAGGTAACGGTCGTTGCCGCCTTC

AGTGCAATGCATG 3' (SEQ ID NO:73)
5'TCACGTTAC (SEQ ID NO:74)

The 1-2 and 3-4 pairs (fragments 1-4) were ligated together over a two hour time period, as were pairs 5-6 and 7-8 to form two double-stranded sequences. The ligation product of fragments 1-4 was digested with Nco I and Nar I, whereas the product of fragments 5-8 was digested with Nar I and Sph I. These digestions separated any concatamers formed during ligation and provided the necessary sticky ends for further ligation.

The digested mixes were run on 6 percent acrylamide gels. The bands of correct size were excised from the gels, and the DNA was eluted from the gel matrix.

The DNA fragments of (1-4) and (5-6) were ligated together to form a 177 base pair molecule. As above, the ligation was digested with restriction enzymes to create the necessary ends for subsequent cloning of the molecule. In this case, the ligation of fragments (1-4) and (5-8) was digested with Nco I and Sph I. The digested ligation product DNA segment was run on a 6 percent polyacrylamide gel. The band of 177 base pairs was excised and eluted from the gel.

The 177 base pair fragment was cloned into plasmid pARC466. Plasmid pARC466 is a plasmid identical to M13mp19 except that an Nco I site has

-141-

replaced the native Hind III site. This plasmid contains a polylinker region including a Sma I site that is downstream from the Sph I site.

5 The Nco I site in plasmid pARC466 was created by replacing the originally present Hind III site using in vitro mutagenesis as discussed previously. The primer used was:

10 5' CCT GCA GGC ATC CAA CCA TGG CGT
(SEQ ID NO:75)
AAT CAT GGT CAT 3'

15 Plasmid pARC466 was digested with Nco I and Sph I. The 177 bp transit peptide DNA fragment ends were designed to clone into these sites. The ligation of the 177 base pair fragment into plasmid pARC466 resulted in plasmid pARC480. Plasmid pARC480 was sequenced by M13 protocol to check the sequence of the designed peptide, which sequence was found to
20 be correct.

ii. Plasmid pATC212

The transit peptide was moved into a plasmid that contained a plant promoter and termination
25 sequence. pCaMVCN is a plasmid supplied by Pharmacia that contains the 35S promoter and a NOS polyadenylation sequence. The transit peptide was cloned next to the 35S promoter as follows:

30 a) Plasmid pCaMVCN was digested with the restriction enzyme Sal I. Linker #1104 from New England Biolabs d(TCGACCCGGG) was digested with Sal I and then ligated with the digested pCaMVCN to create plasmid pATC209.

35 b) Plasmid pATC209 was digested with Sma I. Plasmid pARC480 was digested with Nco I and Sma I to remove the transit peptide. The Nco I site

-142-

of the transit peptide DNA was treated with the Klenow fragment of E. coli DNA polymerase to create a blunt end to make that fragment compatible with the Sma I site of plasmid pATC209. The double blunt-ended fragment was cloned into the Sma I-digested plasmid pATC209 to create plasmid pATC212.

iii. Plasmid pATC1616

Plasmid pATC1616 is a derivative of plasmid pGA482 that contains the gene for phytoene dehydrogenase-4H with the transit peptide sequence in frame with the coding sequence of the phytoene dehydrogenase-4H gene. This gene construct is driven by the CaMV 35S promoter and contains the NOS polyadenylation site downstream of the structural gene. The plasmid was made in the following way.

The plasmid pATC1607 (Example 11) contains a version of the phytoene dehydrogenase-4H with a Sph I site at the initiation methionine codon. Plasmid pATC1607 was digested with Nco I. The cleaved Nco I site is the same as the Nco I site at position 6342 in Figure 5 and is the Nco I site at about position 1510 in Figure 11. The Nco I site was made blunt by treating with the Klenow fragment of DNA polymerase.

The thus treated plasmid pATC1607 plasmid was then digested with Sph I. This digestion caused the production of about 1506 bp fragment, which includes the structural gene for phytoene dehydrogenase-4H. At the 5' end of the fragment is a Sph I site and at the 3' end of the fragment is a blunt end.

Plasmid pATC212 was digested with Sph I and Sma I. The Sph I site is at the 3' end of the transit peptide sequence and the Sma I site is downstream in the polylinker sequence of the plasmid

-143-

pATC212. The above Sph I-blunt ended phytoene dehydrogenase-4H gene fragment was cloned into the pATC212 plasmid, resulting in plasmid pATC1612.

5 Plasmid pATC1612 contains the CaMV 35S promoter, the transit peptide sequence, the structural phytoene dehydrogenase-4H gene, and the NOS polyadenylation sequence. This whole region of pATC1612 can be moved as an Xba I-Xba I fragment, since there are Xba I sites upstream from the CaMV
10 35S promoter and downstream from the NOS polyadenylation sequence.

Plasmid pATC1612 was digested with Xba I and the about 2450 bp Xba I-Xba I fragment (450 bp CaMV
15 35S promoter, 177 bp transit peptide sequence, 1506 bp phytoene dehydrogenase-4H gene, and the 300 bp NOS polyadenylation sequence) was cloned into the Xba I site of plasmid pGA482. The resulting plasmid is pATC1616.

20 b. Production in the Plant Cytoplasm

To prepare lycopene in the cytoplasm, the carotenoid genes described before are introduced into appropriate vector(s), as also described above for
25 chloroplasts, using identical techniques, except that the transit peptide is eliminated. Because they are not targeted to the chloroplast, the enzymes remain in the cytoplasm, and, acting on the ubiquitous isoprenoid intermediate, farnesyl pyrophosphate, produce lycopene in the cytosol.

30 Example 15. Lycopene Cyclase Gene

a. Localization

The location of the lycopene cyclase gene on pARC376 was established as described before for the
35 other enzyme genes. If the gene for lycopene cyclase

-144-

were deleted, mutated or otherwise impaired, there would not be an active lycopene cyclase enzyme and lycopene would accumulate. Lycopene imparts a red color to E. coli cells producing it, whereas beta-carotene imparts a yellow color to E. coli cells producing beta-carotene.

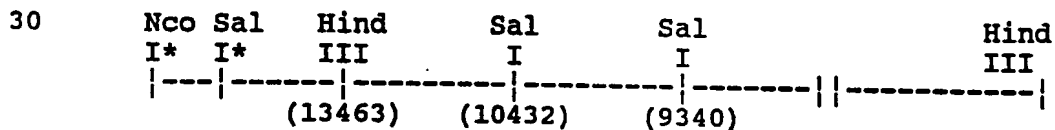
The following experiments demonstrated that the gene is located on a 1548 bp DNA fragment of plasmid pARC376 bounded by the Sal I site (9340) and the Pst I site (7792) shown in Figure 5.

Plasmid pARC376 was partially digested with Ava I, the ends were religated, and the plasmid DNA was transformed into E. coli cells strain HB101. This plasmid, named pARC376-Ava 102, contained a 611 bp Ava I fragment deletion from position 8231 to 8842 and also a 1611 bp Ava I fragment deletion from position 8842 to 10453.

Some E. coli cells transformed with the Ava I digested pARC376 plasmid were found to have impaired lycopene cyclase gene function, and therefore, accumulated lycopene. These results indicated that the gene for lycopene cyclase was present in the region near the Sal I site at 9340.

25 b. Plasmid pARC1009

Example 8b describes the construction of plasmid pARC137B, whose Erwinia herbicola DNA insert is diagrammatically illustrated below.



35 The Nco I and Sal I sites in the above diagram with asterisks are in the polylinker portion of parent plasmid pARC306A.

-145-

Plasmid pARC137B was digested with Sal I and then the region from the polylinker Sal I site to the Sal I site at about original position 9340 was ligated back together, to form plasmid pARC137-5. A Sal I-Sal I fragment of about 4123 bp was thereby removed. The formed plasmid retained the Rec 7 promoter that was now adjacent to the Erwinia herbicola DNA beginning at about the Sal I site at about original position 9340.

The resulting plasmid also contained two Stu I restriction sites between the remaining Sal I and Hind III sites. Those Stu I sites were at about original positions 7306 and 3538.

Digestion of plasmid pARC137-5 with Stu I, and religation of the Stu I-terminated fragments containing the above-illustrated Nco I and Hind III sites resulted in a new plasmid named pARC1009. That plasmid contained Erwinia herbicola DNA of interest from the Sal I site originally at about position 9340 to the Stu I site originally at about position 7306, and the Rec 7 promoter adjacent to that Sal I site.

Plasmid pARC1009 was transformed into E. coli, strain JM101, and the cells were grown and treated with nalidixic acid to induce the Rec 7 promoter. The protein fraction was isolated, analyzed on PAGE and a dominant protein band of 36 kilodaltons was noted. This protein band was identified as the enzyme lycopene cyclase, as discussed hereinafter. The protein band was isolated and subjected to N-terminal amino acid sequencing. The first 25 N-terminal amino acid residues were determined as shown in Figure 19.

Comparison of the N-terminal amino acid sequence of the lycopene cyclase enzyme with the DNA sequence of the pARC376 plasmid revealed the position

-146-

of the initiation codon of the lycopene cyclase gene. Surprisingly, the initiation codon is GTG, not the much more common ATG. A GTG codon normally codes for the amino acid valine, but under rare instances in bacteria, it can also code for methionine when it is the first amino acid in a protein (G.D. Stormo, 1986, in Maximizing Gene Expression, W. Reznikoff, L. Gold (Eds) Butterworths, Stoneham, Massachusetts, pp 195-224.) Thus, from this comparison, the 5' end of the gene for lycopene cyclase was found to begin about 338 bp downstream from the Sal I site at original position 9340.

c. Plasmid pARC465

A series of studies was performed to determine the location of the 3' end of the gene. A plasmid, pARC465, which contains the carotenoid genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and the chloramphenicol acetyltransferase gene that confers resistance to the antibiotic chloramphenicol, was constructed as follows.

The plasmid pARC307D is an analogous plasmid to the plasmid pUC8, except that plasmid pARC307D contains the chloramphenicol acetyltransferase gene instead of the ampicillinase gene. Plasmid pARC307D also contains the same polycloning linker as pUC8.

Plasmid pARC307D was digested with Hind III and Eco RI. The plasmid pARC376-Ava 102 (Example 9b) was also digested with Hind III and Eco RI. The resultant about 8000 bp fragment from Hind III (13463) to Eco RI (3370) of plasmid pARC376-Ava 102 was isolated from an agarose gel (the fragment size is only about 8000 bp because the Ava I deletions in plasmid pARC376-Ava 102 described before deleted

-147-

about 2200 bp from the parent pARC376 plasmid). This about 8000 bp Hind III-Eco RI fragment was cloned into the Hind III- and Eco RI-digested plasmid pARC307D. The resulting plasmid, pARC465, caused the
5 production of lycopene when transformed into E. coli, and also conferred resistance to the antibiotic chloramphenicol.

The plasmid pARC1009, which contains the gene for lycopene cyclase, was introduced into E. coli
10 cells containing plasmid pARC465, and the cells were grown on chloramphenicol and ampicillin. These cells produced beta-carotene. This indicated that the 3' end of the gene for lycopene cyclase was upstream from the Stu I site (original position about 7306).

15

d. Plasmid pARC1008

To further define the location of the 3' end of the gene, the 1548 bp Sal I (9340) to Pst I (7792) DNA fragment (Example 15a) was cloned into plasmid
20 pARC306A. The resulting plasmid, pARC1008, was introduced into E. coli cells that already contained plasmid pARC465. These cells, grown in the presence of chloramphenicol and ampicillin, produced beta-carotene. These results indicated that the 3' end of
25 the gene was present upstream from the Pst I (7792) site.

In summary then, the gene for lycopene cyclase is contained in an about 1548 bp Sal I to Pst I fragment of plasmid pARC376. The actual initiation
30 codon is about 338 bp downstream from the Sal I site. Therefore, the bounds of the gene for lycopene cyclase are approximately from position 9002 to the Pst I site at position 7792 in Figure 5, enclosing an approximately 1210 bp DNA segment. Figure 19
35 contains a nucleotide sequence obtained from single

-148-

strand sequencing and a partial amino acid sequence obtained by amino acid sequencing for lycopene cyclase.

5 Several constructs have been made in which the 5' end of the gene for lycopene cyclase has been modified. Two are described below.

e. Plasmid pARC147

10 In one construct, the initiation codon was changed from a GTG sequence to an ATG sequence by introducing a Nco I site by in vitro mutagenesis at the beginning of the gene as follows. An oligonucleotide probe was synthesized that had the following sequence as compared with the normal
15 sequence:

Native DNA Sequence

20 A GAG CGT ATC GTG AGG GAT CTG ATT TTA GTC GGC G
*MET (SEQ ID NO:76)

New DNA Sequence

25 G CGC GGA TCC ATG GGG GAT CTG ATT TTA GTC GGC G
*MET
Nco I (SEQ ID NO:77)

30 * Initiation Methionine; bold-faced letters are as described before.

35 The Nco I restriction site sequence is CC ATGG, therefore, the new sequence at the initiation methionine introduced an Nco I site. This newly modified lycopene cyclase gene, starting at the introduced Nco I site was cloned into the plasmid pARC306A to generate the plasmid pARC147. Plasmid pARC147 was introduced into E. coli cells already containing plasmid pARC465, and the cells were grown

-149-

in the presence of chloramphenicol and ampicillin. These cells produced beta-carotene. Thus, a functional lycopene cyclase gene within an about 1210 bp DNA fragment from Nco I to Pst I that can be moved
5 into other plasmids for the expression of the enzyme, was constructed.

f. Lycopene Cyclase Assay

Cultured E. coli cells separately transformed
10 with plasmid pARC1606, described below, that cause lycopene accumulation in E. coli, and with plasmid pARC147, discussed before, that contains the Rec 7-driven lycopene cyclase gene were separately homogenized. The homogenates were mixed at a ratio
15 of 1:1 in the presence of 2.5 mM MgCl₂, 3 mM MnCl₂, 4 mM dithiothreitol, and 6 mM ATP for six hours at 30°C.

The assay mixture was thereafter lyophilized and extracted with acetone:methanol (7:2, v:v). The
20 extract was concentrated and analyzed by HPLC. β -Carotene was detected; about 54 ng of the cis isomer and about 27 ng of the trans isomer. Thus, the genetically engineered gene for lycopene cyclase present in plasmid pARC147, was actively transcribed
25 by the transformed E. coli host cells.

Cofactors such as FAD, NADP and FMN are not required for lycopene cyclase activity. ATP is, however, essential for activity.

30 Construction of pARC1606

The construction of plasmid pARC1606 proceeded with a series of intermediate vectors.

The plasmid pARC376 was partially digested with Bam HI and then religated. The religated
35 plasmid was transformed into E. coli cells and cells

-150-

5 were selected that contained a plasmid in which Bam HI fragments of about 1045 bp (from original position 3442 to 4487) and of about 815 bp (from original position 5302 to 4487) were deleted from the pARC376 plasmid. The name of the new plasmid was pARC376-Bam 100, and the plasmid caused the E. coli cells to produce β -carotene, since the gene for β -carotene hydroxylase was deleted.

10 The plasmid pARC376-Bam 100 was digested with Hind III and Eco RI. The fragment containing the Erwinia herbicola carotenoid genes was isolated and religated. The coordinates for the Hind III and Eco RI sites originally from plasmid pARC376 are 13463 and 3370, respectively.

15 Plasmid pARC307D, supra, also contains the pUC8 MCS. Plasmid pARC307D was digested with Hind III and Eco RI, and the Erwinia herbicola Hind III and Eco RI fragment excised from plasmid pARC376-Bam 100 was cloned into plasmid pARC307D to form plasmid pARC279. This plasmid conferred chloramphenicol resistance to the E. Coli cells and also caused them to produce β -carotene. The plasmid pARC279 contains about 11.7 kb.

25 Plasmid pARC279 was partially digested with Bgl II and Bam HI and then religated to delete specific regions from the pARC279 plasmid that were not necessary for β -carotene production and make the plasmid as small as possible. A clone was found in which the size of the plasmid was about 10 kb (about 30 1.7 kb had been deleted), that conferred chloramphenicol resistance to E. coli and caused the synthesis of β -carotene. That plasmid was named pARC281B.

35 Plasmid pARC1606 was made from plasmid pARC281B by mutagenizing E. coli cells that contained

-151-

plasmid pARC281B with nitrosoguanidine (NTG) according to the following protocol.

The following is the NTG mutagenesis protocol:

- 5 1. E. coli cells containing plasmid pARC281B were grown to log phase - about $3-5 \times 10^8$ cells/ml or Absorbance 0.3-0.6 at 600 nm.
2. The cells were washed twice with phosphate buffer (50 mM, pH 7.0), and
10 then resuspended in 1/10th of the original volume of growth medium.
3. NTG was added to the cells in phosphate buffer to a final concentration of 100 $\mu\text{g/ml}$. The cells were incubated for 1
15 hour at 37°C.
4. The cells were washed three times in phosphate buffer to remove the NTG. The cells were then resuspended in Luria-Broth with 25 $\mu\text{g/ml}$ of chloramphenicol
20 and grown for about 15-18 hours at 37°C.
5. The cells were then diluted and plated on Luria-Broth Medium with 1.5 percent Agar with 25 $\mu\text{g/ml}$ chloramphenicol. A colony
25 was found that produced lycopene as evidenced by the red appearance of the colony. The plasmid contained in that colony was isolated and called plasmid pARC1606.

30 A mutation was induced somewhere in the gene for lycopene cyclase after the nitrosoguanidine treatment that caused the inactivation of the enzyme. This caused the cells to accumulate lycopene, the precursor to β -carotene. Cells that contained the plasmid with this mutation were now red, due to the

-152-

accumulation of lycopene, instead of the β -carotene yellow color.

Cells containing plasmid pARC1606 were used as a source of lycopene for the lycopene cyclase assays described before.

g. Plasmid pARC1509

The new construct, plasmid pARC147, that works effectively in *E. coli*, is not effective in yeast. It appears that the second N-terminal amino acid, which was changed from Arg to Gly by the above procedure, made this gene inactive in yeast. Therefore both 5' and 3' ends of the lycopene cyclase gene were genetically re-engineered as follows.

A Sph I restriction site at the initiation Met codon and a Bam HI restriction site at the 3' end of the gene were introduced into the native sequence by PCR (as described before) using the following probes:

For the Sph I site at the 5' end

Sph I (SEQ ID NO:78)
5' G CGG CGC ATG CGG GAT CTG ATT TTA GTC GGC G 3'

For the Bam HI site at the 3' end

Bam HI (SEQ ID NO:79)
5' CAT CGG ATC CTG TCA GGA AAA TGG TTC AGC 3'

An about 3012 bp fragment from Sal I (9407) to the Nco I site (6395) was excised from the plasmid pARC271D described in Example 8. This fragment was used as the template for the PCR reaction that was performed as described previously.

After PCR, the reaction mixture was digested with Sph I and Bam HI. The about 1142 bp fragment shown in Figure 19, between the first G residue of

-153-

the Sph I (18) site and the first G residue of the Bam HI (1168) site, was isolated on agarose gel as previously described. This about 1142 bp Sph I-Bam HI fragment of the cyclase gene was cloned into pUC18 which had been previously digested with Sph I and Bam HI. The resulting plasmid was called pARC1509.

h. Plasmid pARC1510

To determine whether the genetically engineered version of the lycopene cyclase gene in plasmid pARC1509 codes for an active protein, the structural gene segment was introduced adjacent to the TAC promoter in the plasmid pKK223-3 (Pharmacia) as follows. Upstream from the Sph I site of plasmid pARC1509 (in the polycloning sequence) is a unique Hind III site. The plasmid pARC1509 was digested with Hind III and Bam HI, an about 1156 bp Hind III-Bam HI fragment was isolated. The fragment ends were made blunt by treatment with the Klenow fragment of DNA Polymerase I.

The plasmid pKK223-3 contains a unique Eco RI site adjacent to the TAC promoter. Plasmid pKK223-3 was digested with Eco RI and the ends were likewise blunted with the Klenow reagent. The fragment containing the structural gene segment for lycopene cyclase was ligated into the blunted Eco RI site adjacent to the TAC promoter to produce the plasmid pARC1510.

To verify that the gene for lycopene cyclase was capable of expressing an active protein, plasmid pARC1510 was introduced into E. coli cells that already contained the plasmid pARC465 that contains the CAT resistance gene and the genes necessary to produce lycopene, but from which the gene for lycopene cyclase had been deleted. E. coli cells

-154-

containing both plasmids pARC465 and pARC1510, were grown with both chloramphenicol and ampicillin, and produced beta-carotene.

5 **Example 16. Beta-carotene production in *E. coli***

a. Method One - Plasmid(s) containing engineered genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase

10 Four carotenoid enzyme genes are required to produce beta-carotene from ubiquitous precursors, i.e., the genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase. In one example, the first three genes, i.e., for GGPP
15 synthase, phytoene synthase, and phytoene dehydrogenase-4H enzymes, were present on the plasmid pARC465. This plasmid also contains the chloramphenicol acetyltransferase gene that confers resistance to the antibiotic chloramphenicol in
20 *E. coli*.

 The plasmid pARC1009, described in Example 15, contains the about 2038 bp Sal I to Stu I DNA fragment inserted into plasmid pARC306A. When plasmid pARC1009 was transferred to *E. coli* cells
25 that contained the plasmid pARC465, the cells produced beta-carotene at a level of about 0.05 percent (dry weight).

 The plasmid pARC147, also described in Example 15, contains the about 1215 bp Nco I to Pst I
30 fragment that was inserted into the pARC306A plasmid. This plasmid was also introduced into *E. coli* cells that contained the plasmid pARC465, and those cells also synthesized beta-carotene at a level of about 0.05 percent (dry weight). Because it was
35 subsequently discovered that this version of the

-155-

lycopene cyclase structural gene was inactive in yeast, its use was discontinued and the gene was altered as described in Example 15 to produce plasmid pARC1510. Plasmid pARC1510, transferred in combination with plasmid pARC465, produced beta-carotene in E. coli.

b. Alternate Method - Plasmid pARC376 with a defective gene for beta-carotene hydroxylase

The plasmid pARC376 has a sufficient gene complement to effectuate the synthesis of carotenoids up to and including zeaxanthin diglucoside in E. coli. Beta-carotene is the metabolic substrate for the beta-carotene hydroxylase enzyme that adds two hydroxyl groups at the 3 and 3' positions of beta-carotene to produce zeaxanthin. If the gene for beta-carotene hydroxylase is deleted, mutated, or in some other way made non-functional, the cells accumulate the substrate beta-carotene.

i. Plasmid pARC376-Pst 102

The gene for beta-carotene hydroxylase is contained on a 975 bp DNA fragment bounded by a Pst I site (4886) and the Sma I site (5861) in plasmid pARC376. To delete part of the gene for this enzyme, plasmid pARC376 was partially digested with Pst I, and the appropriate cut ends were religated. Analysis of the plasmid DNA determined that the 392 bp Pst I fragment from original position 4886 to 5215 was deleted. This plasmid was named pARC376-Pst 102.

After transformation of plasmid pARC376-Pst 102 into E. coli, colonies with an orange-yellow color were picked and analyzed for carotenoid content by methods described before. The normal color of E. coli colonies containing the intact pARC376

-156-

5 plasmid and producing zeaxanthin diglucoside is yellow. Analysis of the orange-yellow colored colonies revealed that only beta-carotene was being produced at a level of about 0.1 percent (dry weight).

ii. Plasmid pARC376-Bam 100

10 In an analogous procedure, plasmid pARC376 was partially digested with Bam HI and appropriately religated, causing the deletion of an approximately 815 bp fragment from about original position 4487 to 5302. The resultant plasmid was called pARC376-Bam 100. The plasmid DNA was transformed into E. coli HB101, and orange-yellow colonies were selected and
15 analyzed for carotenoid content. Beta-carotene accumulated in these cells at a level of about 0.1 percent.

20 Example 17. Production of beta-carotene in S. cerevisiae

The structural gene for each of the four enzymes required for beta-carotene synthesis is placed adjacent to an appropriate promoter and termination sequence that will properly function in
25 S. cerevisiae. Appropriate promoters include the GAL 1 and GAL 10 divergent promoters, described in the Detailed Description and Example 5, and the phosphoglyceric acid kinase gene promoter (PGK), likewise described. An appropriate terminator is the
30 termination sequence from the PGK gene.

The structural genes for GGPP synthase and phytoene synthase are present in the plasmid pARC145G, adjacent to the GAL 10 and GAL 1 promoters as described in Example 5. The termination sequence
35 from the PGK gene is at the 3' end of the gene for

-157-

phytoene synthase. To produce beta-carotene it was necessary to introduce the genes for phytoene dehydrogenase-4H and lycopene cyclase in vectors that direct the expression of these genes in this microorganism.

One approach to induce beta-carotene synthesis in yeast is to insert these two genes into a vector, such as pARC146, that contains the GAL 10 and GAL 1 divergent promoters and introduce the resultant plasmid into S. cerevisiae that already contains plasmid pARC145G. The resulting population has all of the genetic material required to produce beta-carotene in a form that permits high level expression of the genes.

a. Plasmid pARC1520

The plasmid pARC146D (Example 10) already contains the gene for phytoene dehydrogenase-4H adjacent to the GAL 1 promoter. The structural gene for lycopene cyclase described in Example 15 was cloned into plasmid pARC146D adjacent to the GAL 10 promoter as follows:

The plasmid pARC1509, described in Example 15, was digested with Hind III and Bam HI. The about 1156 bp fragment containing the structural gene for lycopene cyclase was isolated and the ends were blunted by treatment with the Klenow fragment of DNA Polymerase I.

Plasmid pARC146D was digested with Eco RI (restriction site is unique in plasmid pARC146D - see Figure 14). The ends of the Eco RI digested plasmid were also blunted and the lycopene cyclase gene was cloned into plasmid pARC146D to produce the plasmid pARC1520. Plasmid pARC1520, therefore, contains the gene for phytoene dehydrogenase-4H adjacent to the

-158-

GAL 1 promoter, the gene for lycopene cyclase adjacent to the GAL 10 promoter, and the URA 3 gene (described before) useful for selection in yeast. Plasmid pARC1520 was introduced into the S.
5 cerevisiae, strain YPH499, which already contained the plasmid pARC145G. Beta-carotene was produced at the level of about 0.01 percent of the dry weight.

10 **Example 18. Production of beta-carotene in Higher Plants.**

a. Chloroplast

Although beta-carotene is synthesized in the chloroplasts of plants, most higher plant species do not accumulate very high levels of it. Carrot roots
15 are among the best accumulators, but even in these the concentration is only about 0.01-0.1 percent (dry weight). The objective, then, is to increase the catalytic activity of lycopene cyclase and thereby the accumulation of beta-carotene.

20 Lycopene production is thought to be the divergence point of carotenoid synthesis. In one branch, lycopene is converted to alpha-carotene that in turn is converted to lutein. Lutein is the carotenoid that accumulates to the highest
25 concentration level of all carotenoids. In the other branch, lycopene is converted to beta-carotene, which does not accumulate to as high a level as the lutein. If the level for the enzyme for lycopene cyclase is increased, however, beta-carotene accumulates to
30 higher levels.

To increase the level of lycopene cyclase in the chloroplast, the following steps are taken. The 177 bp Nco I to Sph I DNA fragment for the tobacco transit peptide sequence of plasmid pATC212 is linked
35 in frame with the lycopene cyclase structural gene

-159-

having a Sph I site at the initiation codon for methionine of plasmid pARC1509. This procedure is analogous to that described in Example 14, except that the 3'-Bam HI site of the lycopene cyclase gene, rather than the 3'-Nco I site, is blunted with the Klenow fragment of DNA polymerase.

The resulting plasmid is cleaved with Xba I, and the Xba I-Xba I fragment containing the CaMV 353 promoter (about 450 bp), the transit peptide (about 177 bp), lycopene cyclase (about 1150 bp), and NOS polyadenylation (about 300 bp) sequences are cloned into plasmid pGA482. That Xba I-Xba I fragment contains about 2077 bp. This plasmid is transformed into Agrobacterium tumefaciens, strain A281.

The relevant features of pGA482 were described in Example 14 and include (i) the left and right borders of the T-DNA sequence, which directs the integration of the DNA sequences between these borders into the plant genome; (ii) the kanamycin resistance gene using the NOS promoter for expression, which allows the selection of kanamycin resistant plants containing the lycopene cyclase gene; and (iii) an origin of replication that allows the replication of pGA482 in Agrobacterium tumefaciens. pGA482 was introduced into Agrobacterium tumefaciens, strain A281.

Subsequently, plants, such as tobacco and alfalfa, are infected with this Agrobacterium. The gene for lycopene cyclase is expressed under the influence of the CaMV 35S promoter and is directed to the chloroplast by the tobacco transit peptide sequence described before. The resultant plants produce an increased amount of lycopene cyclase, which results in a concomitant increased level of production and accumulation of beta-carotene.

-160-

Other carotenoid enzyme-specific genes can also be utilized in conjunction with the lycopene cyclase gene to increase the production and accumulation of beta-carotene. These include genes for GGPP synthase, phytoene synthase, and phytoene dehydrogenase-4H. The introduction of these genes into higher plants involves the same manipulations as described above for lycopene cyclase. The genes are attached to the tobacco transit peptide DNA sequence and are then placed adjacent to a functional plant promoter, such as the CaMV 35S promoter. Also placed adjacent, is a polyadenylation sequence, such as the NOS polyadenylation sequence.

These gene constructs are introduced into plants along with the gene for lycopene cyclase, and the combination results in increased total enzyme activity in this portion of the carotenoid synthesis pathway. This further results in an increase of beta-carotene synthesis and accumulation in the chloroplast.

b. Cytoplasm

Introducing Erwinia herbicola genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase results in beta-carotene synthesis in the cytoplasm. In order to express these enzymes in plant cells, the structural genes are individually cloned into one or more vectors that contain a promoter and a polyadenylation sequence that will function in plants. One such vector is the before-described pCaMVCN, with the CaMV 35S promoter and the NOS polyadenylation sequence. The four genes with the appropriate promoters and polyadenylation signals are then inserted into the before-described plasmid, pGA482.

-161-

Plasmid pGA482, containing the four carotenoid-specific genes with the appropriate regulatory signals, is transformed into A. tumefaciens, such as strain A281. Subsequently, plants such as tobacco and alfalfa are infected with the A. tumefaciens, containing the four carotenoid genes, during which process, the carotenoid genes are transfected and integrated into the plant genome. The result is that the transformed plants have the necessary genes, and the capacity to produce and accumulate beta-carotene in the cytoplasm. The CaMV 35S promoter causes the carotenoid genes to be expressed.

Example 19. β -Carotene Production in Pichia pastoris

The before-described method is also extendable to other yeasts. One yeast system that serves as an example is the methylotrophic yeast, Pichia pastoris.

To produce β -carotene in P. pastoris, structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase are placed under the control of regulatory sequences that direct expression of structural genes in Pichia. The resultant expression-competent forms of those genes are introduced into Pichia cells.

For example, the transformation and expression system described by Cregg et al., Biotechnology 5:479-485 (1987); Molecular and Cellular Biology 12:3376-3385 (1987) can be used. A structural gene for GGPP synthase such as that from plasmid pARC489D is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. Similarly, structural genes for

-162-

phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase such as those from plasmids pARC140N, pARC146D and pARC1509, respectively, are placed between AOX1 promoters and terminators. All four of these genes and their flanking regulatory regions are then introduced into a plasmid that carries both the P. pastoris HIS4 gene and a P. pastoris ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within P. pastoris cells [Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987)].

The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in E. coli cells. The final resultant plasmid carrying GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase genes, as well as the various additional elements described above, is illustratively transformed into a his4 mutant of P. pastoris, i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987), to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the enzymes GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase and the production of β -carotene in P. pastoris.

The four genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase can also be introduced by integrative transformation, which does not require the use of an

-163-

ARS sequence, as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987).

Example 20. β -Carotene Production in *A. nidulans*

5 The genes encoding GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase as discussed before can be used to synthesize and accumulate β -carotene in fungi such as *Aspergillus nidulans*. Genes are transferred to
10 *Aspergillus* by integration.

 For example, the structural gene for GGPP synthase is introduced into the *E. coli* plasmid pBR322. The promoter from a cloned *Aspergillus* gene such as argB [Upshall et al., Mol. Gen. Genet.
15 204:349-354 (1986)] is placed into the plasmid adjacent to the GGPP synthase structural gene. Thus, the GGPP synthase gene is now under the control of the *Aspergillus argB* promoter.

 Next, the entire cloned amds gene [Corrick
20 et al., Gene 53:63-71 (1987)] is introduced into the plasmid. The presence of the amds gene permits acetamide to be used as a sole carbon or nitrogen source, thus providing a means for selecting those *Aspergillus* cells that have become stably transformed
25 with the amds-containing plasmid.

 Thus, the plasmid so prepared contains the *Aspergillus argB* promoter fused to the GGPP synthase gene and the amds gene present for selection of *Aspergillus* transformants. *Aspergillus* is then
30 transformed with this plasmid according to the method of Ballance et al., Biochem. Biophys. Res. Commun. 112:284-289 (1983).

 The GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and lycopene cyclase
35 structural genes are each similarly introduced into

-164-

the E. coli plasmid pBR322. Promoters for the cloned Aspergillus argB gene [Upshall et al., Mol. Gen. Genet., 204:349-354 (1986)] are placed immediately adjacent to those three structural genes. Thus, these structural genes are controlled by the Aspergillus argB promoters.

The entire, cloned Aspergillus trpC gene [Hamer and Timberlake, Mol. Cell. Biol., 7:2352-2359 (1987)] is introduced into the plasmid. The trpC gene permits selection of the integrated plasmid by virtue of permitting transformed trpC mutant Aspergillus cells to now grow in the absence of tryptophan. The Aspergillus strain, already transformed with the plasmid containing the GGPP synthase gene, is now capable of synthesizing β -carotene.

Example 21. Production of Zeaxanthin in E. coli

a. Construction of Plasmid pARC404BH

The about 2938 bp fragment from the Eco RV site at position 4323 to the Stu I site at position 7306 from plasmid pARC376 (Figure 5) was cloned into the Sma I site of M13mp19 (obtained from BRL). The resulting plasmid was named pARC404BH-B. This plasmid was used for the introduction of an Nco I site at the initiation methionine of the β -carotene hydroxylase enzyme (position 4991 of Figure 5) using the method described in Ausabel et al. and discussed in Example 2(f).

The oligonucleotide probe used for the in vitro mutagenesis to introduce the Nco I site was:

5' T TAA ACT ATT TAG TAC CAT GGC GGT GCG CGC TCC TG 3'
(SEQ ID NO:80)

Upon introduction of the Nco I site at the β -carotene hydroxylase initiation methionine, the following

-165-

changes occurred in the nucleotide sequence of the enzyme.

Original sequence:

5 CAG GAG CGC GCA CCG CT ATG CTA GTA AAT AGT TTA A... (SEQ ID NO:81)
Met Leu Val Asn Ser Leu ... (SEQ ID NO:82)

New Sequence:

10 CAG GAG CGC GCA CCG CC Nco I ATG GTA CTA AAT AGT TTA A... (SEQ ID NO:83)
Met Val Leu Asn Ser Leu ... (SEQ ID NO:84)

15 The plasmid with the newly introduced Nco I site at the initiation methionine residue was named plasmid pARC404BH-C.

20 Plasmid pARC404BH-C was digested with Nco I and Bam HI. The about 311 bp Nco I to Bam HI fragment (original coordinates from plasmid pARC376 is 4991 for the newly introduced Nco I site and 5302 for the Bam HI site) was isolated and cloned into the Nco I and Bam HI sites of plasmid pARC466 of Example 25 14. This plasmid was called pARC404BH-A.

30 Plasmid pARC376 was digested with Bam HI and Sma I, and the about 559 bp fragment from Bam HI (5302 of Figure 5) to Sma I (5861 of Figure 5) was isolated. The plasmid pARC404BH-A was digested with Bam HI and Sma I.

35 The about 559 bp Bam HI-Sma I fragment isolated from plasmid pARC376 was cloned into the Bam HI and Sma I sites of plasmid pARC404BH-A. The resulting plasmid was called pARC404BH. This plasmid contains the structural gene for β -carotene hydroxylase with the newly introduced Nco I site at the beginning of the gene, whose sequence is included in Figure 21. The structural gene can be moved as an about 870 bp Nco I-Sma I fragment.

-166-

b. E. Coli Production of Zeaxanthin

Plasmid pARC404BH was digested with Nco I and Sma I, and the about 870 bp Nco I-Sma I fragment was isolated. The plasmid pARC306A shown
5 schematically in Figure 6, which contains the Rec 7 promoter, was digested with Nco I and Sma I. The about 870 bp Nco I-Sma I fragment was cloned into the pARC306A plasmid to form to form plasmid pARC406BH. This plasmid contains the structural gene for
10 β -carotene hydroxylase adjacent to the E. coli Rec 7 promoter.

The following study was carried out to show that plasmid pARC406BH encodes a functional β -carotene hydroxylase enzyme. E. coli cells
15 containing the plasmid pARC279 produce β -carotene, but not zeaxanthin, because the gene for β -carotene hydroxylase has been deleted from the plasmid; Example 15(f). Plasmid pARC279 also contains the chloramphenicol acetyltransferase gene that confers
20 resistance to E. coli cells to the antibiotic chloramphenicol. E. coli cells containing the plasmid pARC279 were further transformed with the plasmid pARC406BH, and then the cells were grown in the presence of chloramphenicol and ampicillin.
25 Pigments were analyzed and zeaxanthin was found. This demonstrated that the gene for β -carotene hydroxylase present in plasmid pARC406BH is an active gene that produces a functionally active enzyme.

The structural gene for β -carotene
30 hydroxylase can be moved from plasmid pARC406BH as an about 870 bp Nco I-Sma I fragment. However, there are restriction sites downstream from the Sma I site in the multiple cloning sequence of plasmid pARC306A that also can be used to move the structural gene
35 from plasmid pARC406BH. The structural gene was

-167-

moved as a Nco I-Hind III fragment using one of those downstream restriction sites in the construction of the plasmid pARC145H that is described below.

5 **Example 22.** **Construction of Plasmid pARC145H and
the use of Plasmid pARC145H for
Zeaxanthin Production in Yeast**

Plasmid pARC145G contains the structural
genes for GGPP synthase and phytoene synthase
10 adjacent to the GAL 1 and GAL 10 promoters (Figure
10). There is a unique Sph I site in plasmid
pARC145G near the PGK terminator. The gene for β -
carotene hydroxylase coupled with the yeast PGK
promoter and the termination sequence from the URA 3
15 gene was cloned into the Sph I site of plasmid
pARC145G. The resulting plasmid, pARC145H, contained
three carotenoid genes with GGPP synthase and
phytoene synthase genes using the Gal 10/1 promoters
and the β -carotene hydroxylase gene using the PGK
20 promoter. This plasmid transformed in yeast cells
along with the plasmid pARC1520 which contains the
genes for phytoene dehydrogenase and lycopene cyclase
caused the transformed S. cerevisiae cells to produce
zeaxanthin through the conversion of β -carotene to
25 zeaxanthin by the β -carotene hydroxylase enzyme.

The plasmid pARC145H was constructed
through a series of intermediate vectors as described
below.

30 **b. Isolation of the PGK Promoter**

The gene for 3-phosphoglycerate kinase
(PGK) from Saccharomyces cerevisiae was isolated from
a lambda MG14 library, which was provided by
Dr. Maynard Olsen (Washington University, St. Louis,
35 MO). The strategy to isolate the gene used the

-168-

nucleotide sequence of the PGK gene which had been determined and published [Hitzeman et al., Nucleic Acids Res., 10(23):7791-7808 (1983)]. An oligonucleotide probe was constructed for the region surrounding the initiation methionine of the PGK enzyme. The probe was as follows:

5'ATA AAG ACA TTG TTT TTA GAT CTG TTG TAA 3' (SEQ ID NO: 100)

Two nucleotides were changed from the original sequence and those are shown above in bold. With those two changes, a restriction site for Bgl II (AGATCT) was made.

The lambda MG14 library was screened by hybridization with the above oligonucleotide probe. A lambda clone was found that contained the PGK gene. A 2.6 kb Hind III-Eco RI fragment was excised from lambda clone and cloned into the M13mp19 DNA which had been digested with Hind III and Eco RI. The name of this resulting plasmid was mARC127. The 2.6 kb Hind III to Eco RI fragment contains all of the PGK promoter and part of the structural portion of the PGK gene. (This construct is illustrated in the restriction map on page 7795 of the above Hitzeman et al. article.)

c. Introduction of Bgl II and Eco RI sites in the PGK Promoter

To make a version of the PGK promoter that could be used to express heterologous structural genes, two restriction sites were introduced into the PGK promoter region. These introduced restriction sites enabled the PGK promoter to be moved as a Eco RI and Bgl II fragment.

The Bgl II restriction site was introduced 12 bp upstream from the initiation methionine of the PGK gene. The in vitro mutagenesis protocol of

-169-

Ausabel et al. discussed previously was used to introduce the Bgl II site in the PGK promoter. The plasmid mARC127 was used as the starting DNA source for the modification. The same oligonucleotide probe as listed above for the isolation of the PGK gene was used to introduce a Bgl II site into the PGK promoter. The nucleotide sequence of the native PGK sequence is as follows:

5' TTA CAA CAA ATA TAA AAA CAA TGT CTT TAT ...
MET (SEQ ID NO:85)

Following the in vitro mutagenesis the nucleotide sequence became

5' TTA CAA CAG ATC TAA AAA CAA TGT CTT TAT ...
MET (SEQ ID NO:86)

in which bold letters indicate the changed bases.

The plasmid with the Bgl II restriction site introduced into the PGK promoter was named mARC128.

The restriction site for Eco RI was then introduced into the mARC128 plasmid 530 nucleotides upstream from the Bgl II restriction site using the same in vitro mutagenesis protocol as above. The oligonucleotide probe used for the in vitro mutagenesis protocol was:

5' CTT TAT GAG GGT AAC ATG AAT TCA AGA AGG 3'
(SEQ ID NO:87)

with bold letters as before. The original nucleotide sequence was:

5' CCT TCT TGA ATT GAT GTT ACC CTC ATA AAG 3'
(SEQ ID NO:88)

The nucleotide sequence with the Eco RI site became:

5' CCT TCT TGA AAT CAT GTT ACC CTC ATA AAG 3'
(SEQ ID NO:89)

The plasmid with the Eco RI and the Bgl II restriction sites was named pARC306M. With this

-170-

plasmid, the PGK promoter can be moved as an about 530 bp Eco RI-Bgl II fragment.

d. Construction of Plasmid pARC135A

5 Plasmid pUC18 was digested with Sma I, leaving blunted ends. Nco I linkers (CCCATGGG, obtained from New England Biolabs) were ligated to the Sma I site of the digested pUC18 plasmid, and the plasmid was recycled. The resulting plasmid
10 pSOC109 contained an Nco I site where the Sma I site was originally.

Plasmid pSOC109 was digested with Eco RI and Nco I. The plasmid pARC306M was digested with Bgl II, the ends were polished using the Klenow
15 fragment of DNA Polymerase to form a blunt end, and then an Nco I linker was ligated to the blunted Bgl II position, as before, and the plasmid was recycled.

The treated, recycled plasmid pARC306M
20 was then digested with Eco RI and Nco I. The about 530 bp PGK promoter with Eco RI and Nco I ends was isolated from an agarose gel. This PKG promoter-containing fragment was then cloned into the Eco RI and Nco I digested pSOC109. The resulting plasmid
25 was called pARC135A.

e. Construction of Plasmid pARC300T

A 67 bp oligonucleotide was chemically synthesized for the URA 3 gene terminator. A Hind
30 III site was placed at the 5' end of the terminator and a Kpn I site was placed at the 3' end of the terminator. The sequence of this Hind III-Kpn I fragment is shown below, with underlined restriction sites:

35

-171-

Hind III

5' AGCTTCGAAGAACGAAGGAAGGAGCACAGACTTAGATTGGTATATATACGCATATT
AGCTTCTTGCTTCCTTCCTGGTGTCTGAATCTAACCATATATATGCGTATAA

5

Kpn I

GCGGCCGCGGTAC 3' (SEQ ID NO:90)

CGCCGGCGC (SEQ ID NO:91)

10

15

This Hind III-Kpn I fragment was cloned into the Hind III and Kpn I sites of the plasmid pARC300E to form the plasmid pARC300M. The plasmid pARC300E is a derivative of the plasmid pUC8 with a unique Sma I site between the Aat II and Cla I sites of the plasmid. The restriction map for restriction sites that are present only once in pARC300E is shown in Figure 22, whereas a similar map for plasmid pARC300M is illustrated in Figure 23.

20

A LEU 2 gene not relevant for the present construct was cloned as a blunted fragment into the Sma I site of plasmid pARC300M to form the plasmid pARC300T. A restriction map for plasmid pARC300T similar to those of Figures 22 and 23 is shown in Figure 24.

f. Construction of Plasmid pARC426BH

25

30

The plasmid pARC300T was digested with Eco RI and Hind III. The plasmid pARC135A was digested with Eco RI and Nco I, and the about 530 bp PGK promoter fragment was isolated. The plasmid pARC406BH was digested with Nco I and Hind III and the Nco I-Hind III fragment containing the structural gene for β -carotene hydroxylase was isolated. A tri-ligation was performed with the PGK promoter, the β -carotene hydroxylase structural gene, and the plasmid pARC307T. Graphically this is shown below:

35

Eco RI	Eco RI	Nco I	Nco I	Hind III	Hind III	Kpn I
----	-----	-----	-----	-----	-----	-----
pARC300T	<u>PGK</u> Promoter		β -carotene hydroxylase		<u>URA 3</u> Terminator	pARC300T

-172-

Following the tri-ligation, the resulting plasmid pARC426BH contained the PGK promoter driving the β -carotene hydroxylase gene with the URA 3 terminator at the 3' end of the gene. This cassette could be moved as an about 1500 bp Eco RI-Kpn I fragment into other yeast vectors for expression of this gene in yeast.

g. Construction of Plasmid pARC145H

Plasmid pARC426BH was digested with Eco RI and Kpn I and the about 1500 bp fragment was isolated (about 530 bp for the PGK promoter, about 900 bp for the β -carotene hydroxylase gene, and about 67 bp for the URA 3 terminator). The ends were made blunt by treatment with the Klenow fragment of DNA Polymerase. Sph I linkers (GGCATCC, New England Biolabs) were ligated to the about 1500 bp fragment. The fragment was then digested with Sph I. This Sph I-digested fragment was then cloned into the unique Sph I site of plasmid pARC145G (Figure 10), which contains the genes for GGPP synthase and phytoene synthase to form the plasmid pARC145H.

h. Production of Zeaxanthin in Saccharomyces Cerevisiae

Two plasmids were introduced into the S. Cerevisiae yeast strain YPH499: the plasmid pARC145H, which contains the genes for GGPP synthase, phytoene synthase, and β -carotene hydroxylase; and the plasmid pARC1520, which contains the genes for phytoene dehydrogenase-4H and lycopene cyclase. Doubly transformed yeast cells were grown in the presence of galactose to induce the Gal 10 and Gal 1 promoters.

Under these conditions the transformed yeast cells produced zeaxanthin at about 0.01 percent of the dry cell weight. The gene for β -carotene hydroxylase

-173-

was expressed using the PGK promoter and the enzyme was able to convert β -carotene into zeaxanthin. This study therefore demonstrated that zeaxanthin could be produced in yeast.

5

Example 23. Zeaxanthin Production in *Pichia pastoris*

The before-described method is also extendable to other yeasts. One yeast system that serves as an example is the methylotrophic yeast, *Pichia pastoris*.

10

To produce zeaxanthin in *P. pastoris*, structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase are placed under the control of regulatory sequences that direct expression of structural genes in *Pichia*. The resultant expression-competent forms of those genes are introduced into *Pichia* cells.

15

For example, the transformation and expression system described by Cregg et al., Biotechnology 5:479-485 (1987); Molecular and Cellular Biology 12:3376-3385 (1987) can be used. A structural gene for GGPP synthase such as that from plasmid pARC489D is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. Similarly, structural genes for phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase and beta-carotene hydroxylase such as those from plasmids pARC140N, pARC146D, pARC1509, pARC145H and pARC406BH, respectively, are placed between AOX1 promoters and terminators. All five of these genes and their flanking regulatory regions are then introduced into a plasmid that carries both the *P. pastoris* HIS4 gene and a *P. pastoris* ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within *P. pastoris* cells [Cregg et

20

25

30

35

-174-

al., Molecular and Cellular Biology, 12:3376-3385 (1987)].

5 The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in E. coli cells. The final resultant plasmid carrying GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase genes, as well as the various additional elements described above, is illustratively transformed
10 into a his4 mutant of P. pastoris, i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking
15 histidine, but containing methanol as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987), to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the enzymes GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase and the production
20 of zeaxanthin in P. pastoris.

The five genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase can also be introduced by
25 integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987).

Example 24. Zeaxanthin Production in A. nidulans

30 The genes encoding GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase as discussed before can be used to synthesize and accumulate zeaxanthin in fungi such as Aspergillus nidulans. Genes are transferred to
35 Aspergillus by integration.

-175-

For example, the structural gene for GGPP synthase is introduced into the E. coli plasmid pBR322. The promoter from a cloned Aspergillus gene such as argB [Upshall et al., Mol. Gen. Genet. 204:349-354 (1986)] is placed into the plasmid adjacent to the GGPP synthase structural gene. Thus, the GGPP synthase gene is now under the control of the Aspergillus argB promoter.

Next, the entire cloned amds gene [Corrick et al., Gene 53:63-71 (1987)] is introduced into the plasmid. The presence of the amds gene permits acetamide to be used as a sole carbon or nitrogen source, thus providing a means for selecting those Aspergillus cells that have become stably transformed with the amds-containing plasmid.

Thus, the plasmid so prepared contains the Aspergillus argB promoter fused to the GGPP synthase gene and the amds gene present for selection of Aspergillus transformants. Aspergillus is then transformed with this plasmid according to the method of Ballance et al., Biochem. Biophys. Res. Commun. 112:284-289 (1983).

The GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase and beta-carotene hydroxylase structural genes are each similarly introduced into the E. coli plasmid pBR322. Promoters for the cloned Aspergillus argB gene [Upshall et al., Mol. Gen. Genet. 204:349-354 (1986)] are placed immediately adjacent to those five structural genes. Thus, these structural genes are controlled by the Aspergillus argB promoters.

The entire, cloned Aspergillus trpC gene [Hamer and Timberlake, Mol. Cell. Biol., 7:2352-2359 (1987)] is introduced into the plasmid. The trpC gene permits selection of the integrated plasmid by virtue of permitting transformed trpC mutant Aspergillus cells to

-176-

now grow in the absence of tryptophan. The Aspergillus strain, already transformed with the plasmid containing the GGPP synthase gene, is now capable of synthesizing zeaxanthin.

5

Example 25. Beta-Carotene Hydroxylase in Higher Plants

Higher plants have the genes encoding the enzymes required for carotenoid production and so inherently have the ability to produce carotenoids. Zeaxanthin normally is not accumulated, however, because zeaxanthin so produced in most plants is further converted to other products. The carotenoid-specific genes from Erwinia herbicola described can be used to express beta-carotene hydroxylase for use by the plant as well as to improve accumulation of zeaxanthin in plants. Two useful approaches are described below.

10

15

a. Transport to the Chloroplast

In the first approach, the gene for beta-carotene hydroxylase of Figure 21 is modified to introduce the restriction site Sph I in place of the Nco I site shown in Figure 21 at the initiation methionine codon, using the method of Ausabel et al. as discussed before. This change of the Nco I to a Sph I site changes the second amino acid residue in the expressed enzyme variant to be a leucine residue, so that the first three residues of the enzyme have the sequence Met-Leu-Leu.

20

25

Following the procedure of Example 14, the plasmid containing the engineered Sph I site is cleaved with Sma I and the Sph I to yield a fragment of about 870 bp containing the beta-carotene hydroxylase structural gene. Plasmid pATC212 that contains the CaMV 35S promoter, the transit peptide and NOS site is cleaved with Sph I and Sma I and the above about 870 bp

30

35

-177-

fragment is cloned into the respective sites to form a plasmid analogous to plasmid pATC1612.

Cleavage of the above plasmid with Xba I provides an Xba I-Xba I fragment that includes about 1797 bp. The Xba I-Xba I fragment contains the following: the about 450 bp CaMV 35S promoter, (b) the 177 bp transit peptide sequence, (c) the about 870 bp beta-carotene hydroxylase gene, and (d) the about 300 bp NOS polyadenylation sequence.

This Xba I-Xba I gene construct is inserted into the plasmid pGA482 (Pharmacia) in a Xba I restriction site within the multiple cloning linker region to form a plasmid. The relevant features of pGA482 include (i) an origin of replication that permits maintenance of the plasmid in Agrobacterium tumefaciens, (ii) the left and right border sequences from the T-DNA region that direct the integration of the DNA segment between the borders into the plant genome, and (iii) the NOS promoter adjacent to the kanamycin resistance gene that permits plant cells to survive in the presence of kanamycin.

The above-formed plasmid is transformed into Agrobacterium tumefaciens LBA4404 (Clontech, Inc.) according to standard protocols. Agrobacterium cells containing the plasmid with the transit peptide-beta-carotene hydroxylase gene construct are transferred by infection of tobacco leaf discs using the method of Horsch et al., Science, 227:1229-1231 (1985). During the infection process, the entire DNA segment between the left and right borders of the original pGA482 plasmid is transfected into the plant cells. Transfected plant cells are selected for kanamycin resistance, grown under usual conditions and accumulate zeaxanthin.

-178-

A transformed plant as described in Example 18a that contains at least the structural gene for lycopene cyclase, and preferably also contains the genes described herein for GGPP synthase, phytoene synthase and phytoene dehydrogenase-4H, is also useful as a host for transformation as described in this example.

b. Production in the Plant Cytoplasm

The carotenoid genes described before are introduced into appropriate vector(s), as also described above for chloroplasts, using similar techniques, except that the transit peptide is eliminated, and the 5' Nco I site shown in Figure 21 is retained. A plasmid containing the beta-carotene hydroxylase gene such as PARC406BH is cleaved with Nco I. The resulting sticky end is made blunt by filling in with the Klenow fragment of DNA polymerase. Cleavage with Sma I provides a double blunt-ended fragment that can be cloned into the Sma I site of plasmid pATC209 to form a plasmid that contains an about 1620 bp fragment excisable with Xba I that can be cloned into a plasmid such as pGA482 and then used to transform A. tumefaciens.

The resulting, transformed A. tumefaciens is then utilized to transform a higher plant such as tobacco or alfalfa such as that produced in Example 18b. The resulting plants have the necessary genes, and the capacity to produce and accumulate zeaxanthin in the cytoplasm due to expression via the CaMV 35S promoter.

Example 26. Production of Zeaxanthin Diglucoside in E. coli

Example 1 illustrated the production of zeaxanthin diglucoside in E. coli transformed with plasmid pARC376. The discussion below describes the

-179-

production of zeaxanthin diglucoside using E. coli transformed with two plasmids.

5 The location of the DNA sequence that encodes zeaxanthin glycosylase in plasmid pARC376 was readily accomplished in view of the work described in the previous examples related to the preceding five structural genes. The glycosylase-encoding structural gene was found to be located just downstream from the Eco RV restriction site at about position 10256 (Figure 10 5). The coding sequence was found to begin with the ATG codon located at position 10232.

PCR primers designed to yield an Nde I site at the 5' end and an Ava I site at the 3' end of the structural gene were prepared. Only three bases in the 15 native sequence had to be changed. The sequences of the PCR primers were as follows:

For the 5' end

Native 5' ATACGCCATGAGCCATTTTGCCATTGTGGC
(SEQ ID NO:92)
20
Primer 5' ATACCATATGAGCCATTTTGCCATTGTGGC 3'
Nde I (SEQ ID NO:93)
25

For the 3' end

Native 5' GCCCCCGGGAGTCAGATCGTCTTCATGGA
(SEQ ID NO:94)
30
Primer 5' GCCCCCGGGAGTCAGATCGTCTTCATGGA 3'
Ava I (SEQ ID NO:95)
35
3'

40 The template utilized for this mutagenesis reaction was obtained from a Bam HI-Bam HI (position 10524-7775 of Figure 5) fragment obtained from plasmid pARC137B (Example 8b). The PCR techniques utilized in Example 8 were utilized here also.

-180-

The PCR product was digested with Nde I and
Ava I to provide an approximately 1390 bp fragment that
was cloned into the Rec 7 promoter plasmid pARC305N
(Example 3) to form plasmid pARC2019. The Ava I site
occurs at position 8842 of plasmid pARC376. Clones were
verified by regenerating the fragment plus vector
pattern on an agarose gel after digestion with Nde I and
Ava I.

E. coli (HB101) that had been transformed with
plasmid pARC288 (Example 1b) and produce zeaxanthin were
further transformed with plasmid pARC2019. The
resulting cells were lysed and the cell extracts
analyzed by thin layer chromatography. Extracts from E.
coli cells transformed with plasmids pARC288 and pARC376
were used as standards. Zeaxanthin diglucoside was
identified in the extracts from E. coli transformed with
both of plasmids pARC288 and pARC2019.

Example 27. Production of Zeaxanthin Diglucoside in
S. cerevisiae

Zeaxanthin diglucoside is produced in the
yeast S. cerevisiae using the multiply transformed
strain YPH499 prepared in Example 22h that is further
transformed with an appropriate vector that can express
zeaxanthin glycosylase in yeast. The preparation of
such a vector is discussed below.

Plasmid pARC2019 is digested with Nde I and
the ends are filled in with the Klenow fragment of DNA
polymerase 1 to form DNA having blunt ends. The
synthetic linker

GAATTC
CTTAAG

is ligated between the two blunt ends.

The resulting circular plasmid is then cleaved
with Ava I. A synthetic linker of the sequence

-181-

CCGGGAATTC (SEQ ID NO:96)
TTAAC

is linked into the cleaved plasmid, and the plasmid is recircularized.

5 The above procedures provide a plasmid having the structural gene for zeaxanthin diglucoside between two Eco RI restriction sites. That plasmid is then cleaved with Eco RI to provide an approximately 1401 bp Eco RI-Eco RI fragment.

10 Plasmid pSOC713 is cleaved with Eco RI, and the above approximately 1401 bp Eco RI-Eco RI fragment is excised and then ligated therein to form a plasmid appropriate for expression of the zeaxanthin glycosylase gene in S. cerevisiae, in which the structural gene
15 encoding zeaxanthin glycosylase is under the control of the GAL 10 promoter. Transformation of the transformed, zeaxanthin-producing S. cerevisiae of Example 22h provides yeasts that produce zeaxanthin diglucoside.

20 **Example 28: Zeaxanthin Diglucoside Production in**
Pichia pastoris

The before-described method is also extendable to other yeasts. One yeast system that serves as an example is the methylotrophic yeast, Pichia pastoris.

25 To produce zeaxanthin diglucoside in P. pastoris, structural genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase are placed under the control of regulatory sequences that
30 direct expression of structural genes in Pichia. The resultant expression-competent forms of those genes are introduced into Pichia cells.

For example, the transformation and expression system described by Cregg et al., Biotechnology 5:479-485 (1987); Molecular and Cellular Biology 12:3376-3385
35 (1987) can be used. A structural gene for GGPP synthase

-182-

such as that from plasmid pARC489D is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. Similarly, structural genes for phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and zeaxanthin glycosylase, such as those from plasmids pARC140N, pARC146D, pARC1509, pARC145H, pARC4068H and pARC2019, respectively, are placed between AOX1 promoters and terminators. All six of these genes and their flanking regulatory regions are then introduced into a plasmid that carries both the P. pastoris HIS4 gene and a P. pastoris ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within P. pastoris cells [Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987)].

The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in E. coli cells. The final resultant plasmid carrying GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase genes, as well as the various additional elements described above, is illustratively transformed into a his4 mutant of P. pastoris, i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987), to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the enzymes GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin

-183-

glycosylase and the production of zeaxanthin diglucoside in P. pastoris.

The six genes for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al., Molecular and Cellular Biology, 12:3376-3385 (1987).

Example 29. Zeaxanthin Diglucoside Production in A. nidulans

The genes encoding GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and zeaxanthin glycosylase as discussed before can be used to synthesize and accumulate zeaxanthin diglucoside in fungi such as Aspergillus nidulans. Genes are transferred to Aspergillus by integration.

For example, the structural gene for GGPP synthase is introduced into the E. coli plasmid pBR322. The promoter from a cloned Aspergillus gene such as argB [Upshall et al., Mol. Gen. Genet. 204:349-354 (1986)] is placed into the plasmid adjacent to the GGPP synthase structural gene. Thus, the GGPP synthase gene is now under the control of the Aspergillus argB promoter.

Next, the entire cloned amds gene [Corrick et al., Gene 53:63-71 (1987)] is introduced into the plasmid. The presence of the amds gene permits acetamide to be used as a sole carbon or nitrogen source, thus providing a means for selecting those Aspergillus cells that have become stably transformed with the amds-containing plasmid.

Thus, the plasmid so prepared contains the Aspergillus argB promoter fused to the GGPP synthase

-184-

gene and the amds gene present for selection of Aspergillus transformants. Aspergillus is then transformed with this plasmid according to the method of Ballance et al., Biochem. Biophys. Res. Commun. 112:284-289 (1983).

The GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase and zeaxanthin glycosylase structural genes are each similarly introduced into the E. coli plasmid pBR322. Promoters for the cloned Aspergillus argB gene [Upshall et al., Mol. Gen. Genet., 204:349-354 (1986)] are placed immediately adjacent to those six structural genes. Thus, these structural genes are controlled by the Aspergillus argB promoters.

The entire, cloned Aspergillus trpC gene [Hamer and Timberlake, Mol. Cell. Biol., 7:2352-2359 (1987)] is introduced into the plasmid. The trpC gene permits selection of the integrated plasmid by virtue of permitting transformed trpC mutant Aspergillus cells to now grow in the absence of tryptophan. The Aspergillus strain, already transformed with the plasmid containing the GGPP synthase gene, is now capable of synthesizing zeaxanthin diglucoside.

Example 30. Zeaxanthin Glycosylase and Zeaxanthin Diglucoside in Higher Plants

Higher plants such as tobacco and alfalfa have the genes encoding the enzymes required for carotenoid production and so inherently have the ability to produce carotenoids. Zeaxanthin diglucoside normally is not accumulated, however, because zeaxanthin diglucoside so produced in most plants is further converted to other products. The carotenoid-specific genes from Erwinia herbicola described can be used to express zeaxanthin glycosylase for use by the plant as well as to improve

-185-

accumulation of zeaxanthin diglucoside in plants. Two useful approaches are described below.

a. Transport to the Chloroplast

5 In the first approach, the structural gene for zeaxanthin glycosylase of Figure 25 is modified to introduce the restriction site Sph I instead of the Nde I site shown in Figure 25 at the initiation methionine codon, using a method of Ausabel et al. as discussed
10 before. This process, using plasmid pARC2019, for example, changes the second amino acid residue of the enzyme from a serine to an arginine, but provides a biologically active zeaxanthin glycosylase enzyme variant.

15 Following the procedures of Example 14, the plasmid containing the engineered Sph I site is cleaved first with Ava I and made blunt-ended with the Kenow fragment of DNA polymerase. Cleavage thereafter with Sph I provides an about 1200 bp fragment containing the
20 zeaxanthin glycosylase structural gene. Plasmid pATC212 that contains the CaMV 35S promoter, the transit peptide and NOS polyadenylation site is cleaved with Sph I and Sma I, and the above about 1200 bp fragment is cloned into those respective sites to form a plasmid analogous
25 to plasmid pATC1612.

Cleavage of the above plasmid with Xba I provides an Xba I-Xba I fragment that includes about 2127 bp. That Xba I-Xba I fragment includes the following: (a) the about 450 bp CaMV 35S promoter, (b)
30 the 177 bp transit peptide sequence, (c) the about 1200 bp zeaxanthin glycosylase gene, and (d) the about 300 bp NOS polyadenylation sequence.

This Xba I-Xba I gene construct is inserted into the plasmid pGA482 (Pharmacia) in a convenient
35 restriction site within the multiple cloning linker

-186-

region to form a plasmid. The relevant features of pGA482 include (i) an origin of replication that permits maintenance of the plasmid in Agrobacterium tumefaciens, (ii) the left and right border sequences from the T-DNA region that direct the integration of the DNA segment between the borders into the plant genome, and (iii) the NOS promoter adjacent to the kanamycin resistance gene that permits plant cells to survive in the presence of kanamycin.

The above-formed plasmid is transformed into Agrobacterium tumefaciens LBA4404 (Clontech, Inc.) according to standard protocols. Agrobacterium cells containing the plasmid with the transit peptide-zeaxanthin glycosylase gene construct are transferred by infection of tobacco leaf discs using the method of Horsch et al., Science, 227:1229-1231 (1985). During the infection process, the entire DNA segment between the left and right borders of the original pGA482 plasmid is transfected into the plant cells. Transfected plant cells are selected for kanamycin resistance, grown under usual conditions and accumulate zeaxanthin.

A transformed plant as described in Example 25a that contains at least the structural gene for lycopene cyclase, and preferably also contains the genes described herein for GGPP synthase, phytoene synthase, phytoene dehydrogenase-4H and β -carotene hydroxylase is also useful as a host for transformation as described herein.

b. Production in the Plant Cytoplasm

The carotenoid genes described before are introduced into appropriate vector(s), as also described above for chloroplasts, using similar techniques, except that the transit peptide is eliminated, and the 5' Nde I

-187-

site shown in Figure 25 is retained. A plasmid containing the zeaxanthin glycosylase structural gene such as pARC2019 is cleaved with Nde I and Ava I. The resulting sticky ends are made blunt with the Klenow
5 fragment of DNA polymerase. The resulting double blunt-ended is cloned into the Sma I site of plasmid pARC209 to form a plasmid that contains an Xba I-excisable fragment that includes the CaMV 35S promoter, zeaxanthin glycosylase gene and NOS polyadenylation sequence that
10 can be cloned into a plasmid such as pGA482 that is then used to transform A. tumefaciens.

The resulting, transformed A. tumefaciens is then utilized to transform a higher plant such as tobacco or alfalfa such as that produced in Example 25b.
15 The resulting plants have the necessary genes, and the capacity to produce and accumulate zeaxanthin diglucoside in the cytoplasm due to expression via the CaMV 35S promoter.

Although the present invention has now been
20 described in terms of certain preferred embodiments, and exemplified with respect thereto, one skilled in the art will readily appreciate that various modifications, changes, omissions and substitutions may be made without departing from the spirit thereof. It is intended,
25 therefore, that the present invention be limited solely by the scope of the following claims.

-188-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ausich, Rodney L
Brinkhaus, Friedhelm L
Mukharji, Indrani
Proffitt, John H
Yarger, James G
Yen, Huei-Che B

(ii) TITLE OF INVENTION: Biosynthesis of Zeaxanthin and
Glycosylated Zeaxanthin in
Genetically Engineered Hosts

(iii) NUMBER OF SEQUENCES: 100

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Amoco Corp., Patents and Licensing Dept
(B) STREET: 200 E Randolph St
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: USA
(F) ZIP: 60680-0703

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Galloway, Norval B

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 3128567180
(B) TELEFAX: 3128564972

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1157 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-189-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGA -121

TCTAAAGGCA CAGCGTCTCA TGCTTCGCAC AATGTAAAAC TGCTTCAGAA CCTGGCGAGA	-61
GCTATCCGCG CGGTCTACGG TTAAGTATA CTAAAAGACA ATTCAGCGGG TAACCTTGCA	-1
ATGGTGAGTG GCAGTAAAGC GGGCGTTTCG CCTCATCGCG AAATAGAGGT AATGAGACAA	60
TCCATTGACG ATCACCCTGGC TGGCCTGTTA CCTGAAACCG ACAGCCAGGA TATCGTCAGC	120
CTTGCGATGC GTGAAGGCGT CATGGCACCC GGTAAACGGA TCCGTCCGCT GCTGATGCTG	180
CTGGCCGCCC GCGACCTCCG CTACCAGGGC AGTATGCCTA CGCTGCTCGA TCTCGCCTGC	240
GCCGTTGAAC TGACCCATAC CGCGTCGCTG ATGCTCGACG ACATGCCCTG CATGGACACC	300
GCCGAGCTGC GCCGCGGTCA GCCCACTACC CACAAAAAAT TTGGTGAGAG CGTGGCGATC	360
CTTGCCTCCG TTGGGCTGCT CTCTAAAGCC TTTGGTCTGA TCGCCGCCAC CGGCGATCTG	420
CCGGGGGAGA GCGGTGCCCA GCGGTCAAC GAGCTCTCTA CCGCCGTGGG GCTGCAGGGC	480
CTGGTACTGG GGCAGTTTCG CGATCTTAAC GATGCCGCCC TCGACCGTAC CCCTGACGCT	540
ATCCTCAGCA CCAACCACCT CAAGACCGGC ATTCTGTTCA GCGCGATGCT GCAGATCGTC	600
GCCATTGCTT CCGCCTCGTC GCCGAGCAGC CGAGAGACGC TGCACGCCCT CGCCCTCGAC	660
TTGGGCCAGG CGTTTCAACT GCTGGACGAT CTGCGTGACG ATCACCCGGA AACCGGTAAA	720
GATCGCAATA AGGACGCGGG AAAATCGACG CTGGTCAACC GGCTGGGCGC AGACGCGGGC	780
CGGCAAAAGC TGC GCGAGCA TATTGATTCC GCCGACAAAC ACCTCACTTT TGCCTGTCCG	840
CAGGGCGGCG CCATCCGACA GTTTATGCAT CTGTGGTTTG GCCATCACCT TGCCGACTGG	900
TCACCGGTCA TGAAAATCGC CTGATACCGC CCTTTTGGGT TCAAGCAGTA CATAACGATG	960
GAACCACATT ACAGGAGTAG TGATGAATGA AGGACGAGCG CCTTGTTTCAG CGTAAGAACG	1020
ATCATCTGGA TATC	1034

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

-190-

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Ser Gly Ser Lys Ala Gly Val Ser Pro His Arg Glu Ile Glu
 1 5 10 15
 Val Met Arg Gln Ser Ile Asp Asp His Leu Ala Gly Leu Leu Pro Glu
 20 25 30
 Thr Asp Ser Gln Asp Ile Val Ser Leu Ala Met Arg Glu Gly Val Met
 35 40 45
 Ala Pro Gly Lys Arg Ile Arg Pro Leu Leu Met Leu Leu Ala Ala Arg
 50 55 60
 Asp Leu Arg Tyr Gln Gly Ser Met Pro Thr Leu Leu Asp Leu Ala Cys
 65 70 75 80
 Ala Val Glu Leu Thr His Thr Ala Ser Leu Met Leu Asp Asp Met Pro
 85 90 95
 Cys Met Asp Asn Ala Glu Leu Arg Arg Gly Gln Pro Thr Thr His Lys
 100 105 110
 Lys Phe Gly Glu Ser Val Ala Ile Leu Ala Ser Val Gly Leu Leu Ser
 115 120 125
 Lys Ala Phe Gly Leu Ile Ala Ala Thr Gly Asp Leu Pro Gly Glu Arg
 130 135 140
 Arg Ala Gln Ala Val Asn Glu Leu Ser Thr Ala Val Gly Leu Gln Gly
 145 150 155 160
 Leu Val Leu Gly Gln Phe Arg Asp Leu Asn Asp Ala Ala Leu Asp Arg
 165 170 175
 Thr Pro Asp Ala Ile Leu Ser Thr Asn His Leu Lys Thr Gly Ile Leu
 180 185 190
 Phe Ser Ala Met Leu Gln Ile Val Ala Ile Ala Ser Ala Ser Ser Pro
 195 200 205
 Ser Thr Arg Glu Thr Leu His Ala Phe Ala Leu Asp Phe Gly Gln Ala
 210 215 220
 Phe Gln Leu Leu Asp Asp Leu Arg Asp Asp His Pro Glu Thr Gly Lys
 225 230 235 240
 Asp Arg Asn Lys Asp Ala Gly Lys Ser Thr Leu Val Asn Arg Leu Gly
 245 250 255

-191-

Ala Asp Ala Ala Arg Gln Lys Leu Arg Glu His Ile Asp Ser Ala Asp
 260 265 270

Lys His Leu Thr Phe Ala Cys Pro Gln Gly Gly Ala Ile Arg Gln Phe
 275 280 285

Met His Leu Trp Phe Gly His His Leu Ala Asp Trp Ser Pro Val Met
 290 295 300

Lys Ile Ala
 305

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1157 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGATCTAAAG GCACAGCGTC TCATGCTTCG -121

CACAATGTAA AACTGCTTCA GAACCTGGCG AGAGCTATCC GCGCGGTCTA CGGTAACTG -61

ATACTAAAAG ACAATTCAGC GGGTAACCTT GCAATGGTGA GTGGCAGTAA AGCGGGCGTC -1

ATGGCCGAAT TCGAAATAGA GGTAATGAGA CAATCCATTG ACGATCACCT GGCTGGCCTG 60

TTACCTGAAA CCGACAGCCA GGATATCGTC AGCCTTGCGA TCGTGAAAG CGTCATGGCA 120

CCCGGTAAAC GGATCCGTCC GCTGCTGATG CTGCTGGCCG CCCGCGACCT CCGCTACCAG 180

GGCAGTATGC CTACGCTGCT CGATCTCGCC TGCGCCGTTG AACTGACCCA TACCGCGTCG 240

CTGATGCTCG ACGACATGCC CTGCATGGAC ACCGCCGAGC TGCGCCGCGG TCAGCCCACT 300

ACCCACAAAA AATTTGGTGA GAGCGTGGCG ATCCTTGCCCT CCGTTGGGCT GCTCTCTAAA 360

GCCTTTGGTC TGATCGCCGC CACCGGCGAT CTGCCGGGGG AGAGGCGTGC CCAGGCGGTC 420

AACGAGCTCT CTACCGCCGT GGGGCTGCAG GGCCTGGTAC TGGGGCAGTT TCGCGATCTT 480

AACGATGCCG CCCTCGACCG TACCCCTGAC GCTATCCTCA GCACCAACCA CCTCAAGACC 540

GGCATTCTGT TCAGCGCGAT GCTGCAGATC GTCGCCATTG CTTCCGCCTC GTCGCCGAGC 600

ACGCGAGAGA CGCTGCACGC CTTGCCCTC GACTTCGGCC AGGCGTTTCA ACTGCTGGAC 660

-192-

GATCTGCGTG ACGATCACCC GGAAACCGGT AAAGATCGCA ATAAGGACGC GGGAAAATCG 720
 ACGCTGGTCA ACCGGCTGGG CGCAGACGCG GCCCGGCAAA AGCTGCGCGA GCATATTGAT 780
 TCCGCCGACA AACACCTCAC TTTTGCCTGT CCGCAGGGCG GCGCCATCCG ACAGTTTATG 840
 CATCTGTGGT TTGGCCATCA CCTTGCCGAC TGGTCACCGG TCATGAAAAT CGCCTGATAC 900
 CGCCCTTTTG GGTTC AAGCA GTACATAACG ATGGAACCAC ATTACAGGAG TAGTGATGAA 960
 TGAAGGACGA GCGCCTTGTT CAGCGTAAGA ACGATCATCT GGATATC 1007

2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 298 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Glu Phe Glu Ile Glu Val Met Arg Gln Ser Ile Asp Asp His
 1 5 10 15
 Leu Ala Gly Leu Leu Pro Glu Thr Asp Ser Gln Asp Ile Val Ser Leu
 20 25 30
 Ala Met Arg Glu Gly Val Met Ala Pro Gly Lys Arg Ile Arg Pro Leu
 35 40 45
 Leu Met Leu Leu Ala Ala Arg Asp Leu Arg Tyr Gln Gly Ser Met Pro
 50 55 60
 Thr Leu Leu Asp Leu Ala Cys Ala Val Glu Leu Thr His Thr Ala Ser
 65 70 75 80
 Leu Met Leu Asp Asp Met Pro Cys Met Asp Asn Ala Glu Leu Arg Arg
 85 90 95
 Gly Gln Pro Thr Thr His Lys Lys Phe Gly Glu Ser Val Ala Ile Leu
 100 105 110
 Ala Ser Val Gly Leu Leu Ser Lys Ala Phe Gly Leu Ile Ala Ala Thr
 115 120 125
 Gly Asp Leu Pro Gly Glu Arg Arg Ala Gln Ala Val Asn Glu Leu Ser
 130 135 140

-193-

Thr	Ala	Val	Gly	Leu	Gln	Gly	Leu	Val	Leu	Gly	Gln	Phe	Arg	Asp	Leu
145					150					155					160
Asn	Asp	Ala	Ala	Leu	Asp	Arg	Thr	Pro	Asp	Ala	Ile	Leu	Ser	Thr	Asn
				165					170					175	
His	Leu	Lys	Thr	Gly	Ile	Leu	Phe	Ser	Ala	Met	Leu	Gln	Ile	Val	Ala
			180					185					190		
Ile	Ala	Ser	Ala	Ser	Ser	Pro	Ser	Thr	Arg	Glu	Thr	Leu	His	Ala	Phe
		195					200					205			
Ala	Leu	Asp	Phe	Gly	Gln	Ala	Phe	Gln	Leu	Leu	Asp	Asp	Leu	Arg	Asp
	210					215					220				
Asp	His	Pro	Glu	Thr	Gly	Lys	Asp	Arg	Asn	Lys	Asp	Ala	Gly	Lys	Ser
225					230					235					240
Thr	Leu	Val	Asn	Arg	Leu	Gly	Ala	Asp	Ala	Ala	Arg	Gln	Lys	Leu	Arg
			245						250					255	
Glu	His	Ile	Asp	Ser	Ala	Asp	Lys	His	Leu	Thr	Phe	Ala	Cys	Pro	Gln
			260					265					270		
Gly	Gly	Ala	Ile	Arg	Gln	Phe	Met	His	Leu	Trp	Phe	Gly	His	His	Leu
		275					280					285			
Ala	Asp	Trp	Ser	Pro	Val	Met	Lys	Ile	Ala						
	290						295								

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1198 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	GATTG AGGATCTGCA	-1
ATGAGCCAAC	CGCCGCTGCT TGACCACGCC ACGCAGACCA TGGCCAACGG CTCGAAAAGT	60
TTTGCCACCG	CTGCGAAGCT GTTCGACCCG GCCACCCGCC GTAGCGTGCT GATGCTCTAC	120
ACCTGGTGCC	GCCACTGCGA TGACGTCATT GACGACCAGA CCCACGGCTT CGCCAGCGAG	180
GCCGCGGCGG	AGGAGGAGGC CACCCAGCGC CTGGCCCGGC TGCGCACGCT GACCCTGGCG	240

-194-

```

GCGTTTGAAG GGGCCGAGAT GCAGGATCCG GCCTTCGCTG CCTTTCAGGA GGTGGCGCTG 300
ACCCACGGTA TTACGCCCCG CATGGCGCTC GATCACCTCG ACGGCTTTGC GATGGACGTG 360
GCTCAGACCC GGTATGTCAC CTTTGAGGAT ACGCTGCGCT ACTGCTATCA CGTGGCGGGC 420
GTGGTGGGTC TGATGATGGC CAGGGTGATG GGCGTGCGGG ATGAGCGGGT GCTGGATCGC 480
GCCTGCGATC TGGGGCTGGC CTTCCAGCTG ACGAATATGG CCCGGGATAT TATTGACGAT 540
GCGGCTATTG ACCGCTGCTA TCTGCCCCGC GAGTGGCTGC AGGATGCCGG GCTGGCCCCG 600
GAGAACTATG CCGCGCGGGA GAATCGCCCC GCGCTGGCGC GGTGGCGGAG GCTTATTGAT 660
GCCGCAGAGC CGTACTACAT CTCCTCCCAG GCCGGGCTAC ACGATCTGCG GCGGCGCTCC 720
GCGTGGGCGA TCGCCACCGC CCGCAGCGTC TACCGGGAGA TCGGTATTAA GGTAAAAGCG 780
GCGGGAGGCA GCGCCTGGGA TCGCCGCCAG CACACCAGCA AAGGTGAAAA AATTGCCATG 840
CTGATGGCGG CACCGGGGCA GGTATTTCGG GCGAAGACGA CGAGGGTGAC GCCGCGTCCG 900
GCCGGTCTTT GGCAGCGTCC CGTTTAGGCG GGCGGCCATG ACGTTCACGC AGGATCGCCT 960
GTAGGTCGGC AGGCTTGCGG GCGTAAATAA AACC GAAGGA GACGCAGCCC TCCCGGCCGC 1020
GCACCGCGTG GTGCAGGCGG TGGGCGACGT AGAGCCGCTT CAGGTAGCCC CGGCGCGGGA 1080
TCCAGTGGA A GGGCCAGCGC TGATGCACCA GACCGTCGTG CACCAGGAAG TAGAGCAGGC 1140
CATAGACCGT CATGCCGCG CCAATCCACT GCAGGGGCCA AAC 1183

```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 308 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Ser Gln Pro Pro Leu Leu Asp His Ala Thr Gln Thr Met Ala Asn 1
1          5          10          15
Gly Ser Lys Ser Phe Ala Thr Ala Ala Lys Leu Phe Asp Pro Ala Thr 20
20          25          30
Arg Arg Ser Val Leu Met Leu Tyr Thr Trp Cys Arg His Cys Asp Asp 35
35          40          45

```

-195-

Val Ile Asp Asp Gln Thr His Gly Phe Ala Ser Glu Ala Ala Ala Glu
 50 55 60
 Glu Glu Ala Thr Gln Arg Leu Ala Arg Leu Arg Thr Leu Thr Leu Ala
 65 70 75 80
 Ala Phe Glu Gly Ala Glu Met Gln Asp Pro Ala Phe Ala Ala Phe Gln
 85 90 95
 Glu Val Ala Leu Thr His Gly Ile Thr Pro Arg Met Ala Leu Asp His
 100 105 110
 Leu Asp Gly Phe Ala Met Asp Val Ala Gln Thr Arg Tyr Val Thr Phe
 115 120 125
 Glu Asp Thr Leu Arg Tyr Cys Tyr His Val Ala Gly Val Val Gly Leu
 130 135 140
 Met Met Ala Arg Val Met Gly Val Arg Asp Glu Arg Val Leu Asp Arg
 145 150 155 160
 Ala Cys Asp Leu Gly Leu Ala Phe Gln Leu Thr Asn Met Ala Arg Asp
 165 170 175
 Ile Ile Asp Asp Ala Ala Ile Asp Arg Cys Tyr Leu Pro Ala Glu Trp
 180 185 190
 Leu Gln Asp Ala Gly Leu Ala Pro Glu Asn Tyr Ala Ala Arg Glu Asn
 195 200 205
 Arg Pro Ala Leu Ala Arg Trp Arg Arg Leu Ile Asp Ala Ala Glu Pro
 210 215 220
 Tyr Tyr Ile Ser Ser Gln Ala Gly Leu His Asp Leu Arg Arg Arg Ser
 225 230 235 240
 Ala Trp Ala Ile Ala Thr Ala Arg Ser Val Tyr Arg Glu Ile Gly Ile
 245 250 255
 Lys Val Lys Ala Ala Gly Gly Ser Ala Trp Asp Arg Arg Gln His Thr
 260 265 270
 Ser Lys Gly Glu Lys Ile Ala Met Leu Met Ala Ala Pro Gly Gln Val
 275 280 285
 Ile Arg Ala Lys Thr Thr Arg Val Thr Pro Arg Pro Ala Gly Leu Trp
 290 295 300
 Gln Arg Pro Val
 305

-196-

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1518 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

	TAAACC	-1
ATGGAAAAAA CCGTTGTGAT TGGCGCAGGC TTTGGTGGCC TGGCGCTGGC GATTCGCCTG		60
CAGGCGGCAG GGATCCCAAC CGTACTGCTG GAGCAGCGGG ACAAGCCCGG CGGTCGGGGC		120
TACGTCTGGC ATGACCAGGG CTTTACCTTT GACGCCGGGC CGACGGTGAT CACCGATCCT		180
ACCGCGCTTG AGGCGCTGTT CACCCTGGCC GGCAGGCGCA TGGAGGATTA CGTCAGGCTG		240
CTGCCGGTAA AACCCTTCTA CCGACTCTGC TGGGAGTCCG GGAAGACCCT CGACTATGCT		300
AACGACAGCT TCGAGCTTGA GGCAGAGATT ACCCAGTTCA ACCCCGCGA CGTCGAGGGC		360
TACCGGCGCT TTCTGGCTTA CTCCAGGCG GTATTCCAGG AGGGATATTT GCGCCTCGGC		420
AGCGTGCCGT TCCTCTCTTT TCGCGACATG CTGCGCGCCG GGCCGAGCT GCTTAAGCTC		480
CAGGCGTGGC AGAGCGTCTA CCAGTCGGTT TCGCGCTTTA TTGAGGATGA GCATCTGCGG		540
CAGGCCTTCT CGTTCCACTC CCTGCTGGTA GGCGGCAACC CCTTCACCAC CTCGOTCCATC		600
TACACCCTGA TCCACGCCCT TGAGCGGGAG TGGGGGTCT GGTTCCTGA GGGCGGCACC		660
GGGGCGCTGG TGAACGGCAT GGTGAAGCTG TTTACCGATC TGGGCGGGGA GATCGAACTC		720
AACGCCCGGG TCGAAGAGCT GGTGGTGGCC GATAACCGCG TAAGCCAGGT CCGGCTCGCG		780
GATGGTCGGA TCTTTGACAC CGACGCCGTA GCCTCGAACG CTGACGTGGT GAACACCTAT		840
AAAAAGCTGC TCGGCACCAT ACCGGTGGGG CAGAAGCGGG CGGCACGGCT GGAGCGCAAG		900
AGCATGAGCA ACTCGCTGTT TGTGCTCTAC TTCGGCCTGA ACCAGCCTCA TTCCAGCTG		960
GCGCACCATA CCATCTGTTT TGGTCCCCGC TACCGGGAGC TGATCGACGA GATCTTTACC		1020
GGCAGCGCGC TGGCGGATGA CTTCTCGCTC TACCTGCACT CGCCCTGCGT GACCGATCCC		1080
TCGCTCGCGC CTCCCCCGTG CGCCAGCTTC TACGTGCTGG CCCCGGTGCC GCATCTTGGC		1140
AACGCGCCGC TGGACTGGGC GCAGGAGGGG CCGAAGCTGC GCGACCGCAT CTTTGACTAC		1200

-197-

CTTGAAGAGC GCTATATGCC CGGCCTGCGT AGCCAGCTGG TGACCCAGCG GATCTTTACC 1260
 CGGCAGACTT CACGACACGC TTGGATCGCG ATCTTGGGAT CGCTTTTCAT CGAGCCGCCT 1320
 TCGTTGACCC AAGGCTTGTT CGCCGCAAAC GCGACACGAC ATTCAAACCT CTACCTGGTG 1380
 GCCGCAGGTA CTCACCCTGG CGCGGGCATT CCTGGCGTAG TGGGCCTCGC CGAAAGCACC 1440
 GCCAGCCTGA TGATTGAGGA TCTGCAATGA GCCAACCGCC GCTGCTTGAC CACGCCACGC 1500
 AGACCATGGC CA 1512

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 489 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Lys Thr Val Val Ile Gly Ala Gly Phe Gly Gly Leu Ala Leu
 1 5 10 15
 Ala Ile Arg Leu Gln Ala Ala Gly Ile Pro Thr Val Leu Leu Glu Gln
 20 25 30
 Arg Asp Lys Pro Gly Gly Arg Ala Tyr Val Trp His Asp Gln Gly Phe
 35 40 45
 Thr Phe Asp Ala Gly Pro Thr Val Ile Thr Asp Pro Thr Ala Leu Glu
 50 55 60
 Ala Leu Phe Thr Leu Ala Gly Arg Arg Met Glu Asp Tyr Val Arg Leu
 65 70 75 80
 Leu Pro Val Lys Pro Phe Tyr Arg Leu Cys Trp Glu Ser Gly Lys Thr
 85 90 95
 Leu Asp Tyr Ala Asn Asp Ser Phe Glu Leu Glu Ala Gln Ile Thr Gln
 100 105 110
 Phe Asn Pro Arg Asp Val Glu Gly Tyr Arg Arg Phe Leu Ala Tyr Ser
 115 120 125
 Gln Ala Val Phe Gln Glu Gly Tyr Leu Arg Leu Gly Ser Val Pro Phe
 130 135 140

-198-

Leu Ser Phe Arg Asp Met Leu Arg Ala Gly Pro Gln Leu Leu Lys Leu
 145 150 155 160
 Gln Ala Trp Gln Ser Val Tyr Gln Ser Val Ser Arg Phe Ile Glu Asp
 165 170 175
 Glu His Leu Arg Gln Ala Phe Ser Phe His Ser Leu Leu Val Gly Gly
 180 185 190
 Asn Pro Phe Thr Thr Ser Ser Ile Tyr Thr Leu Ile His Ala Leu Glu
 195 200 205
 Arg Glu Trp Gly Val Trp Phe Pro Glu Gly Gly Thr Gly Ala Leu Val
 210 215 220
 Asn Gly Met Val Lys Leu Phe Thr Asp Leu Gly Gly Glu Ile Glu Leu
 225 230 235 240
 Asn Ala Arg Val Glu Glu Leu Val Val Ala Asp Asn Arg Val Ser Gln
 245 250 255
 Val Arg Leu Ala Asp Gly Arg Ile Phe Asp Thr Asp Ala Val Ala Ser
 260 265 270
 Asn Ala Asp Val Val Asn Thr Tyr Lys Lys Leu Leu Gly Thr Ile Pro
 275 280 285
 Val Gly Gln Lys Arg Ala Ala Arg Leu Glu Arg Lys Ser Met Ser Asn
 290 295 300
 Ser Leu Phe Val Leu Tyr Phe Gly Leu Asn Gln Pro His Ser Gln Leu
 305 310 315 320
 Ala His His Thr Ile Cys Phe Gly Pro Arg Tyr Arg Glu Leu Ile Asp
 325 330 335
 Glu Ile Phe Thr Gly Ser Ala Leu Ala Asp Asp Phe Ser Leu Tyr Leu
 340 345 350
 His Ser Pro Cys Val Thr Asp Pro Ser Leu Ala Pro Pro Pro Cys Ala
 355 360 365
 Ser Phe Tyr Val Leu Ala Pro Val Pro His Leu Gly Asn Ala Pro Leu
 370 375 380
 Asp Trp Ala Gln Glu Gly Pro Lys Leu Arg Asp Arg Ile Phe Asp Tyr
 385 390 395 400
 Leu Glu Glu Arg Tyr Met Pro Gly Leu Arg Ser Gln Leu Val Thr Gln
 405 410 415
 Arg Ile Phe Thr Arg Gln Thr Ser Arg His Ala Trp Ile Ala Ile Leu
 420 425 430

-199-

Gly Ser Leu Phe Ile Glu Pro Pro Ser Leu Thr Gln Gly Leu Phe Ala
 435 440 445

Ala Asn Ala Thr Arg His Ser Asn Leu Tyr Leu Val Ala Ala Gly Thr
 450 455 460

His Pro Gly Ala Gly Ile Pro Gly Val Val Gly Leu Ala Glu Ser Thr
 465 470 475 480

Ala Ser Leu Met Ile Glu Asp Leu Gln
 485

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1522 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAGGTCGACG -1

ATGGAAAAAA CCGTTGTGAT TGGCGCAGGC TTTGGTGGCC TGGCGCTGGC GATTCGCCTG 60

CAGGCGGCAG GGATCCCAAC CGTACTGCTG GAGCAGCGGG ACAAGCCCGG CGGTCGGGGC 120

TACGTCTGGC ATGACCAGGG CTTTACCTTT GACGCCGGGC CGACGGTGAT CACCGATCCT 180

ACCGCGCTTG AGGCGCTGTT CACCCTGGCC GGCAGGCGCA TGGAGGATTA CGTCAGGCTG 240

CTGCCGGTAA AACCCTTCTA CCGACTCTGC TGGGAGTCCG GGAAGACCCT CGACTATGCT 300

AACGACAGCT TCGAGCTTGA GGCGCAGATT ACCCAGTTCA ACCCCGCGA CGTCGAGGGC 360

TACCGGCGCT TTCTGGCTTA CTCCCAGGCG GTATTCCAGG AGGGATATTT GCGCCTCGGC 420

AGCGTGCCGT TCCTCTCTTT TCGCGACATG CTGCGCGCCG GGCCGCAGCT GCTTAAGCTC 480

CAGGCGTGGC AGAGCGTCTA CCAGTCGGTT TCGCGCTTTA TTGAGGATGA GCATCTGCGG 540

CAGGCCTTCT CGTTCCACTC CCTGCTGGTA GGCGGCAACC CCTTCACCAC CTCGTCCATC 600

TACACCCTGA TCCACGCCCT TGAGCGGGAG TGGGGGGTCT GGTTCCTGA GGGCGGCACC 660

GGGGCGCTGG TGAACGGCAT GGTGAAGCTG TTTACCGATC TGGGCGGGGA GATCGAACTC 720

AACGCCCCGG TCGAAGAGCT GGTGGTGGCC GATAACCGCG TAAGCCAGGT CCGGCTCGCG 780

-200-

GATGGTCGGA TCTTTGACAC CGACGCCGTA GCCTCGAACG CTGACGTGGT GAACACCTAT 840
 AAAAAGCTGC TCGGCACCAT ACCGGTGGGG CAGAAGCGGG CGGCACGGCT GGAGCGCAAG 900
 AGCATGAGCA ACTCGCTGTT TGTGCTCTAC TTCGGCCTGA ACCAGCCTCA TTCCCAGCTG 960
 GCGCACCATA CCATCTGTTT TGGTCCCCGC TACCGGGAGC TGATCGACGA GATCTTTACC 1020
 GGCAGCGCGC TGGCGGATGA CTTCTCGCTC TACCTGCACT CGCCCTGCGT GACCGATCCC 1080
 TCGCTCGCGC CTCCCCCGTG CGCCAGCTTC TACGTGCTGG CCCCAGTGCC GCATCTTGGC 1140
 AACGCGCCGC TGGACTGGGC GCAGGAGGGG CCGAAGCTGC GCGACCGCAT CTTTGACTAC 1200
 CTTGAAGAGC GCTATATGCC CGGCCTGCGT AGCCAGCTGG TGACCCAGCG GATCTTTACC 1260
 CGGCAGACTT CACGACACGC TTGGATCGCG ATCTTGGGAT CGCTTTTCAT CGAGCCGCCT 1320
 TCGTTGACCC AAGGCTTGTT CGCCGCAAAC GCGACACGAC ATTCAAACCT CTACCTGGTG 1380
 GCCGCAGGTA CTCACCCTGG CGCGGGCATT CCTGGCGTAG TGGGCCTCGC CGAAAGCACC 1440
 GCCAGCCTGA TGATTGAGGA TCTGCAATGA GCCAACCGCC GCTGCTTGAC CACGCCACGT 1500
 CGACCATGGC CA 1512

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 177 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGGCTTCCT CAGTTCTTTC CTCTGCAGCA GTTGCCACCC GCAGCAATGT TGCTCAAGCT 60
 AACATGGTGG CGCCTTTCAC TGGCCTTAAG TCAGCTGCCT CATTCCCTGT TTCAAGGAAG 120
 CAAAACCTTG ACATCACTTC CATTGCCAGC AACGGCGGAA GAGTGCAATG CATGCAG 177

-201-

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1235 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

	CAGGGAGTG AGAGCGTATC	-1
GTGAGGGATC TGATTTTAGT CGGCCGCGGC CTGGCCAACG GGCTGATCGC CTGGCGTCTG		60
CGCCAGCGCT ACCCGCAGCT TAACCTGCTG CTGATCGAGG CCGGGGAGCA GCCCGGCGGG		120
AACCATACCT GGTCATTCCA TGAAGACGAT CTGACTCCCG GGCAGCACGC CTGGCTGGCC		180
CCGCTGGTGG CCCACGCCTG GCCGGGCTAT GAGGTGCAGT TTCCCGATCT TCGCCGTCGC		240
CTCGCGCGCG GCTACTACTC CATTACCTCA GAGCGCTTTG CCGAGGCCCT GCATCAGGCG		300
CTGGGGGAGA ACATCTGGCT AAAGTGTTCG GTGAGCGAGG TGTTACCCAA TAGCGTGCGC		360
CTTGCCAACG GTGAGGCGCT GCTTGCCGGA GCGGTGATTG ACGGACGCGG CGTGACCGCC		420
AGTTCGGCGA TGCAAACCGG CTATCAGCTC TTTCTTGGTC AGCAGTGGCG GCTGACACAG		480
CCCCACGGCC TGACCGTACC GATCCTGATG GATGCCACGG TGGCGCAGCA GCAGGGCTAT		540
CGCTTTGTCT ACACGCTGCC GCTCTCCGCC GACACGCTGC TGATCGAGGA TACGCGCTAC		600
GCCAATGTCC CGCAGCGTGA TGATAATGCC CTACGCCAGA CCGTTACCGA CTATGCTCAC		660
AGCAAAGGGT GGCAGCTGGC CCAGCTTGAA CGCGAGGAGA CCGGCTGTCT GCCGATTACC		720
TGGCGGGTGA CATCCAGGCT CTGTGGGCCG ATGCGCCGGC GTGCCGCGTC GCGAATGCGG		780
GCTGGGCTAT TTCACCCTAC CACTGGCTAT TCGCTGCCGC TGGCGGTGGC CCTTGCCGAC		840
GCGATTGCCG ACAGCCCGCG GCTGGGCAGC GTTCCGCTCT ATCAGCTCAC CCGGCAGTTT		900
GCCGAACGCC ACTGGCGCAG GCAGGGATTG TTCCGCCTGC TGAACCGGAT GCTTTTCCTG		960
GCCGGGCGCG AGGAGAACCG CTGGCGGGTG ATGCAGCGCT TTTATGGGCT GCCGGAGCCC		1020
ACCGTAGAGC GCTTTTACGC CGGTCCGCTC TCTCTCTTTG ATAAGGCCCG CATTTTGACG		1080
GGCAAGCCAC CCGTTCCGCT GCGGAAGTCT GCGGGCGGC GCTGAACCAT TTTCTGACA		1140

-202-

GACGAGATAA AGGATGAAAA AAACCGTTGT GATTGGCGCA GGCTTTGGTG GCCTGGCGCT 1200
 GGCGATTTCG CTGCAG 1216

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1235 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGGGAGTG AGAGGCGCGC -1
 ATGCGGGATC TGATTTTAGT CGGCCGCGGC CTGGCCAACG GGCTGATCGC CTGGCGTCTG 60
 CGCCAGCGCT ACCCGCAGCT TAACCTGCTG CTGATCGAGG CCGGGGAGCA GCCCGGCGGG 120
 AACCATACCT GGTCATTCCA TGAAGACGAT CTGACTCCCG GGCAGCACGC CTGGCTGGCC 180
 CCGCTGGTGG CCCACGCCTG GCCGGGCTAT GAGGTGCAGT TTCCCGATCT TCGCCGTCGC 240
 CTCGCGCGCG GCTACTACTC CATTACCTCA GAGCGCTTTG CCGAGGCCCT GCATCAGGCG 300
 CTGGGGGAGA ACATCTGGCT AAAGTGTTCG GTGAGCGAGG TGTTACCCAA TAGCGTGCGC 360
 CTTGCCAACG GTGAGGCGCT GCTTGCCGGA GCGGTGATTG ACGGACGCGG CGTGACCGCC 420
 AGTTCGGCGA TGCAAACCGG CTATCAGCTC TTTCTTGGTC AGCAGTGGCG GCTGACACAG 480
 CCCCACGGCC TGACCGTACC GATCCTGATG GATGCCACGG TGGCGCAGCA GCAGGGCTAT 540
 CGCTTTGTCT ACACGCTGCC GCTCTCCGCC GACACGCTGC TGATCGAGGA TACGCGCTAC 600
 GCCAATGTCC CGCAGCGTGA TGATAATGCC CTACGCCAGA CCGTTACCGA CTATGCTCAC 660
 AGCAAAGGGT GGCAGCTGGC CCAGCTTGAA CGCGAGGAGA CCGGCTGTCT GCCGATTACC 720
 TGGCGGGTGA CATCCAGGCT CTGTGGGCGG ATGCGCCGGC GTGCCGCGTC GGAATGCGG 780
 GCTGGGCTAT TTCACCCTAC CACTGGCTAT TCGCTGCCGC TGGCGGTGGC CCTTGCCGAC 840
 GCGATTGCCG ACAGCCCGCG GCTGGGCAGC GTTCCGCTCT ATCAGCTCAC CCGGCAGTTT 900
 GCCGAACGCC ACTGGCGCAG GCAGGGATTG TTCCGCCTGC TGAACCGGAT GCTTTTCCTG 960
 GCCGGGCGCG AGGAGAACCG CTGGCGGGTG ATGCAGCGCT TTTATGGGCT GCCGGAGCCC 1020

-203-

ACCGTAGAGC GCTTTTACGC CGGTCGGCTC TCTCTCTTTG ATAAGGCCCG CATTTTGACG 1080
 GGCAAGCCAC CGGTTCCGCT GGCGAAGTCT GGCGGGCGGC GCTGAACCAT TTTCTGACA 1140
 GACGAGATAA AGGGATCCGA TGACCGTTGT GATTGGCGCA GGCTTTGGTG GCCTGGCGCT 1200
 GGCGATTTCG CTGCAG 1216

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 374 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Arg Asp Leu Ile Leu Val Gly Arg Gly Leu Ala Asn Gly Leu Ile
 1 5 10 15
 Ala Trp Arg Leu Arg Gln Arg Tyr Pro Gln Leu Asn Leu Leu Leu Ile
 20 25 30
 Glu Ala Gly Glu Gln Pro Gly Gly Asn His Thr Trp Ser Phe His Glu
 35 40 45
 Asp Asp Leu Thr Pro Gly Gln His Ala Trp Leu Ala Pro Leu Val Ala
 50 55 60
 His Ala Trp Pro Gly Tyr Glu Val Gln Phe Pro Asp Leu Arg Arg Arg
 65 70 75 80
 Leu Ala Arg Gly Tyr Ser Ile Thr Ser Glu Arg Phe Ala Glu Ala
 85 90 95
 Leu His Gln Ala Leu Gly Glu Asn Ile Trp Leu Asn Cys Ser Val Ser
 100 105 110
 Glu Val Leu Pro Asn Ser Val Arg Leu Ala Asn Gly Glu Ala Leu Leu
 115 120 125
 Ala Gly Ala Val Ile Asp Gly Arg Gly Val Thr Ala Ser Ser Ala Met
 130 135 140
 Gln Thr Gly Tyr Gln Leu Phe Leu Gly Gln Gln Trp Arg Leu Thr Gln
 145 150 155 160

[illegible]

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 947 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-205-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

                                     TGAG TCGCAGGAGC GCGCACCGCT   -1
ATGCTAGTAA ATAGTTTAAT CGTCATCTTG ACCGTTATTG CGATGGAGGG CATCGCCGCG   60
TTTACCCACC GCTACATTAT GCACGGCTGG GGATGGCGCT GGCATGAGCC ACACCATACC  120
CCGCGCAAGG GCGTATTTGA GCTAAACGAT CTCTTTGCGG TGGTGTTTGC CGGGGTGGCT  180
ATCGCGCTGA TTGCCGTGGG CACGGCGGGC GTTTGGCCCC TGCAGTGGAT TGGCTGCGGC  240
ATGACGGTCT ATGGCCTGCT CTA CTTCCTG GTGCACGACG GTCTGGTGCA TCAGCGCTGG  300
CCCTTCCACT GGATCCCGCG CCGGGGCTAC CTGAAGCGGC TCTACGTCGC CCACCGCCTG  360
CACCACGCGG TCGCGGGCCG GGAGGGCTGC GTCTCCTTCG GTTTTATTTA CGCCCGCAAG  420
CCTGCCGACC TACAGGCGAT CCTGCGTGAA CGTCATGGCC GCCCGCCTAA ACGGGACGCT  480
GCCAAAGACC GGCCGGACGC GCGGTCACCC TCGTCGTCTT CGCCCGAATA ACCTGCCCCG  540
GTGCCGCCAT CAGCATGGCA ATTTTTTCAC CTTTGCTGGT GTGCTGGCGG CGATCCCAGG  600
CGCTGCCTCC CGCCGCTTTT ACCTTAATAC CGATCTCCCG GTAGACGCTG CGGGCGGTGG  660
CGATCGCCCA CGCGGAGCGC CGCCGCAGAT CGTGTAGCCC GGCCTGGGAG GAGATGTAGT  720
ACGGCTCTGC GGCATCAATA AGCCTCCGCC ACCGCGCCAG CGCGGGGCGA TTCTCCCGCG  780
CGGCATAGTT CTCCGGGGCC AGCCCGGCAT CCTGCAGCCA CTCGGCGGGC AGATAGCAGC  840
GGTCAATAGC CGCATCGTCA ATAATATCCC GGGCCATATT CGTCAGCTGG AAGGCCAGCC  900
CCAGATCGCA GCGCGGATCC AGC                                           923
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 947 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-206-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

                                TGAG TCGCAGGAGC GCGCACCGCC   -1
ATGGTACTAA ATAGTTTAAT CGTCATCTTG ACCGTTATTG CGATGGAGGG CATCGCCGCG   60
TTTACCCACC GCTACATTAT GCACGGCTGG GGATGGCGCT GGCATGAGCC ACACCATAACC  120
CCGCGCAAGG GCGTATTTGA GCTAAACGAT CTCTTTGCGG TGGTGTGTTGC CGGGGTGGCT  180
ATCGCGCTGA TTGCCGTGGG CACGGCGGGC GTTTGGCCCC TGCAGTGGAT TGGCTGCGGC  240
ATGACGGTCT ATGGCCTGCT CTAATTCTTG GTGCACGACG GTCTGGTGCA TCAGCGCTGG  300
CCCTTCCACT GGATCCCGCG CCGGGGCTAC CTGAAGCGGC TCTACGTCGC CCACCGCCTG  360
CACCACGCGG TGC GCGGCCG GGAGGGCTGC GTCTCCTTCG GTTTTATTTA CGCCCGCAAG  420
CCTGCCGACC TACAGGCGAT CCTGCGTGAA CGTCATGGCC GCGCGCCTAA ACGGGACGCT  480
GCCAAAGACC GGCCGGACGC GCGGTCACCC TCGTCGTCTT CGCCCGAATA ACCTGCCCCG  540
GTGCCGCCAT CAGCATGGCA ATTTTTTCAC CTTTGCTGGT GTGCTGGCGG CGATCCCAGG  600
CGCTGCCTCC CGCCGCTTTT ACCTTAATAC CGATCTCCCG GTAGACGCTG CGGGCGGTGG  660
CGATCGCCCA CGCGGAGCGC CGCCGCAGAT CGTGTAGCCC GGCCTGGGAG GAGATGTAGT  720
ACGGCTCTGC GGCATCAATA AGCCTCCGCC ACCGCGCCAG CGCGGGGCGA TTCTCCCGCG  780
CGGCATAGTT CTCCGGGGCC AGCCCGGCAT CCTGCAGCCA CTCGGCGGGC AGATAGCAGC  840
GGTCAATAGC CGCATCGTCA ATAATATCCC GGGCCATATT CGTCAGCTGG AAGGCCAGCC  900
CCAGATCGCA GCGCGGATCC AGC
                                                                923

```

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 176 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Met Leu Val Asn Ser Leu Ile Val Ile Leu Thr Val Ile Ala Met Glu
 1             5             10             15

```

-207-

Gly Ile Ala Ala Phe Thr His Arg Tyr Ile Met His Gly Trp Gly Trp
 20 25 30
 Arg Trp His Glu Pro His His Thr Pro Arg Lys Gly Val Phe Glu Leu
 35 40 45
 Asn Asp Leu Phe Ala Val Val Phe Ala Gly Val Ala Ile Ala Leu Ile
 50 55 60
 Ala Val Gly Thr Ala Gly Val Trp Pro Leu Gln Trp Ile Gly Cys Gly
 65 70 75 80
 Met Thr Val Tyr Gly Leu Leu Tyr Phe Leu Val His Asp Gly Leu Val
 85 90 95
 His Gln Arg Trp Pro Phe His Trp Ile Pro Arg Arg Gly Tyr Leu Lys
 100 105 110
 Arg Leu Tyr Val Ala His Arg Leu His His Ala Val Arg Gly Arg Glu
 115 120 125
 Gly Cys Val Ser Phe Gly Phe Ile Tyr Ala Arg Lys Pro Ala Asp Leu
 130 135 140
 Gln Ala Ile Leu Arg Glu Arg His Gly Arg Pro Pro Lys Arg Asp Ala
 145 150 155 160
 Ala Lys Asp Arg Pro Asp Ala Ala Ser Pro Ser Ser Ser Ser Pro Glu
 165 170 175

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 399 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ser His Phe Ala Ile Val Ala Pro Pro Leu Tyr Ser His Ala Val
 1 5 10 15
 Ala Leu His Ala Leu Ala Leu Glu Met Ala Gln Arg Gly His Arg Val
 20 25 30
 Thr Phe Leu Thr Gly Asn Val Ala Ser Leu Ala Glu Gln Glu Thr Glu
 35 40 45

-208-

Arg Val Ala Phe Tyr Pro Leu Pro Ala Ser Val Gln Gln Ala Gln Arg
 50 55 60
 Asn Val Gln Gln Gln Ser Asn Gly Asn Leu Leu Arg Leu Ile Ala Ala
 65 70 75 80
 Met Ser Ser Leu Thr Asp Val Leu Cys Gln Gln Leu Pro Ala Ile Leu
 85 90 95
 Gln Arg Leu Ala Val Asp Ala Leu Ile Val Asp Glu Met Glu Pro Ala
 100 105 110
 Gly Ser Leu Val Ala Glu Ala Leu Gly Leu Pro Phe Ile Ser Ile Ala
 115 120 125
 Cys Ala Leu Pro Val Asn Arg Glu Leu Pro Leu Pro Val Met Pro Phe
 130 135 140
 His Tyr Ala Glu Asp Lys Arg Ala Arg Ala Arg Phe Gln Val Ser Glu
 145 150 155 160
 Arg Ile Tyr Asp Ala Leu Met Tyr Pro His Gly Gln Thr Ile Leu Arg
 165 170 175
 His Ala Gln Arg Phe Gly Leu Pro Glu Arg Arg Arg Leu Asp Glu Cys
 180 185 190
 Leu Ser Pro Leu Ala Gln Ile Ser Gln Ser Val Pro Ala Leu Asp Phe
 195 200 205
 Pro Arg Arg Ala Leu Pro Asn Cys Phe Thr Tyr Val Gly Ala Leu Arg
 210 215 220
 Tyr Gln Pro Pro Pro Gln Val Glu Arg Ser Pro Arg Ser Thr Pro Arg
 225 230 235 240
 Ile Phe Ala Ser Leu Gly Thr Leu Gln Gly His Arg Leu Arg Leu Phe
 245 250 255
 Gln Lys Ile Ala Arg Ala Cys Ala Ser Val Gly Ala Glu Val Thr Ile
 260 265 270
 Ala His Cys Asp Gly Leu Thr Pro Ala Gln Ala Asp Ser Leu Tyr Cys
 275 280 285
 Gly Ala Thr Glu Val Val Ser Phe Val Asp Gln Pro Arg Tyr Val Ala
 290 295 300
 Glu Ala Asn Leu Val Ile Thr His Gly Gly Leu Asn Thr Val Leu Asp
 305 310 315 320
 Ala Leu Ala Ala Ala Thr Pro Val Leu Ala Val Pro Leu Ser Phe Asp
 325 330 335

-209-

Gln Pro Ala Val Ala Ala Arg Leu Val Tyr Asn Gly Leu Gly Arg Arg
340 345 350

Val Ser Arg Phe Ala Arg Gln Gln Thr Leu Ala Asp Glu Ile Ala Gln
355 360 365

Leu Leu Gly Asp Glu Thr Leu His Gln Arg Val Ala Thr Ala Arg Gln
370 375 380

Gln Leu Asn Asp Ala Gly Gly Thr Pro Arg Cys Gly Asp Pro Asp
385 390 395

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCAGCGGGTA ACCTTGCCAT GGGGAGTGGC AGTAAAGCG 39

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTGCAATGGT GA 12

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-210-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTGCCATGGG GA 12

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CATGGCGAAA TAGAAGCCAT GGGACAATCC ATTGACGAT 39

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAGTAATGAG AC 12

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAGCCATGGG AC 12

-211-

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Val Ser Gly Ser Lys Ala Gly Val Ser Pro His Arg Glu Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Ala Glu Phe Glu Ile
1 5

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATCTAAAAT GAGCCAACCG CCGCTGCTTG ACCACGCCAC GCAGAC 46

(2) INFORMATION FOR SEQ ID NO:27:

-212-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CATGGTCTGC GTGGCGTGGT CAAGCAGCGG CGGTTGGCTC ATTTTA 46

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACAACAAAAT ATAAAAACAA TGTCTTTA 28

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACAACAAGAT CTAAAAACAA TGTCTTTA 28

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-213-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AATTCCCGGG CCATGGC 17

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTGCCATG GCCCGGG 17

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AAACCATGGA AAAAACCGTT GTGATTGGC 29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGCCATGGTC TGC GTGGCGT G 21

-214-

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TAAAGGATGA AAAAAACCGT TGTGATTGGC 30

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met	Lys	Lys	Thr	Val	Val	Ile	Gly
1					5		

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TAAACCATGG AAAAAACCGT TGTGATTGGC 30

(2) INFORMATION FOR SEQ ID NO:37:

-215-

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Glu Lys Thr Val Val Ile Gly
1 5

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GAGATAAAGG ATGAAAAAAA CCGTTGTGAT 30

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GAGGTCGACG ATGAAAAAAA CCGTTGTGAT 30

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-216-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ATGGTCGACG TGGCGTGGTC AAGCAGCGG 29

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GAGATAAAGG ATGAAAAAAA CCGTTGTGAT 30

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met	Lys	Lys	Thr	Val	Val
1				5	

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCATGGAAAA AACCGTTGTG AT 22

-217-

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Glu Lys Thr Val Val
1 5

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GAGGTGACG ATGAAAAAAA CCGTTGTGAT 30

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Met Lys Lys Thr Val Val
1 5

(2) INFORMATION FOR SEQ ID NO:47:

-218-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGCTGCTTG ACCACGCCAC GCAGACCATG G 31

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCGCTGCTTG ACCACGCCAC GTCGACCATG G 31

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GACGAGATAA AGCATGCAAA AAACCGTTGT 30

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

-219-

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Met	Gln	Lys	Thr	Val
1				5

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GACGAGATAA AGGATGAAAA AAACCGTTGT 30

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Met	Lys	Lys	Thr	Val
1				5

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

-220-

GACGAGATAA AGCATGCAAA AAACCGTTGT 30

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Met	Gln	Lys	Thr	Val
1				5

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CATGGCTTCC TCAGTTCTTT CCTCTGCAGC AGTTGCC 37

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

GGGTGGCAAC TGCTGCAGAG GAAAGAACTG AGGAAGC 37

-221-

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ACCCGCAGCA ATGTTGCTCA AGCTAACATG GTGG 34

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CGCCACCATG TTAGCTTGAG CAACATTGCT GC 32

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 50 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CGCCTTTCAC TGGCCTTAAG TCAGCTGCCT CATTCCCTGT TTCAAGGAAG 50

(2) INFORMATION FOR SEQ ID NO:60:

-222-

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 64 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TTTGCTTCCT TGAAACAGGG AATGAGGCAG CGAATGAGGC AGCTGACTTA AGGCCAGTCA 60
AAGG 64

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 54 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CAAAACCTTG ACATCACTTC CATTGCCAGC AACGGCGGAA GAGTGCAATG CATG 54

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 46 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CATTGCACTC TTCCGCCGTT GCTGGCAATG GAAGTGATGT CAAGGT 46

(2) INFORMATION FOR SEQ ID NO:63:

-223-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CATGGCTTCC TCAGTTCTTT CCTCTGCAGC AGTTGCC

37

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GGGTGGCAAC TGCTGCAGAG GAAAGAACTG AGGAAGC 37

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

ACCCGCAGCA ATGTTGCTCA AGCTAACATG GTGG 34

(2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-224-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CGCCACCATG TTAGCTTGAG CAACATTGCT GC 32

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CGCCTTTCAC TGGCCTTAAG TCAGCTGCCT CATTCCCTGT TTCAAGGAAG 50

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TTTGCTTCCT TGAAACAGGG AATGAGGCAG CTGACTTAAG GCCAGTGAAA GG 52

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

CAAAACCTTG ACATCACTTC CATTGCCAGC AACGGCGGAA GAGTGCAATG CATG 54

-225-

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CATTGCACTC TTCCGCCGTT GCTGGCAATG GAAGTGATGT CAAGGT 46

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 71 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CATGGCTTCC TCAGTTCTTT CCTCTGCAGC AGTTGCCACC CGCAGCAATG TTGCTCAAGC 60
TAACATGGTG G 71

(2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 69 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CGCCACCATG TTAGCTTGAG CAACATTGCT GCGGGTGGCA CTGCTGCAG AGGAAAGAAC 60
TGAGGAAGC 69

-226-

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CGCCTTTCAC TGGCCTTAAG TCAGCTGCCT CATTCCCTGT TTCAAGGAAG CAAAACCTTG 60
ACATCACTTC CATTGCCAGC AACGGCGGAA GAGTGCAATG CATG 104

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 98 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CATTGCACTC TCCGCCGTT GCTGGCAATG GAAGTGATGT CAAGGTTTTG CTCCTTGAA 60
ACAGGGAATG AGGCAGCTGA CTTAAGGCCA GTGAAAGG 98

(2) INFORMATION FOR SEQ ID NO:75

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CCTGCAGGCA TCCAACCATG GCGTAATCAT GGT CAT 36

-227-

(2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

AGAGCGTATC GTGAGGGATC TGATTTTAGT CGGCG 35

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GCGCGGATCC ATGGGGGATC TGATTTTAGT CGGCG 35

(2) INFORMATION FOR SEQ ID NO:78:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GCGGCGCATG CGGGATCTGA TTTTAGTCGG CG 32

(2) INFORMATION FOR SEQ ID NO:79:

-228-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CATCGGATCC TGTCAGGAAA ATGGTTCAGC 30

(2) INFORMATION FOR SEQ ID NO:80:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TTAAACTATT TAGTACCATG GCGGTGCGCG CTCCTG 36

(2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CAGGAGCGCG CACCGCTATG CTAGTAAATA GTTTAA 36

(2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

-229-

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Met Leu Val Asn Ser Leu
1 5

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CAGGAGCGCG CACCGCCATG GTACTAAATA GTTTAA 36

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Met Val Leu Asn Ser Leu
1 5

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

-230-

TTACAACAAA TATAAAAACA ATGTCTTTAT 30

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

TTACAACAGA TCTAAAAACA ATGTCTTTAT 30

(2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

CTTTATGAGG GTAACATGAA TTCAAGAAGG 30

(2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CCTTCTTGAA TTGATGTTAC CCTCATAAAG 30

-231-

(2) INFORMATION FOR SEQ ID NO:89:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

CCTTCTTGAA ATCATGTTAC CCTCATAAAG 30

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 69 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

AGCTTCGAAG AACGAAGGAA GGAGCACAGA CTTAGATTGG TATATATACG CATATTGCGG 60
CCGCGGTAC 69

(2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 61 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

AGCTTCTTGC TTCCCTCCTG GTGTCTGAAT CTAACCATAT ATATGCGTAT AACGCCGGCG 60
C 61

-232-

(2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

ATACGCCATG AGCCATTTTG CCATTGTGGC 30

(2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ATACCATATG AGCCATTTTG CCATTGTGGC 30

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

GCCCCCGGG AGTCAGATCG TCTTCATGGA 30

(2) INFORMATION FOR SEQ ID NO:95:

-233-

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GGCCCCCGGG AGTCAGATCG TCTTCATGGA 30

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

CCGGGAATTC 10

(2) INFORMATION FOR SEQ ID NO:97:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

ATGAGCCATT TTGCCATTGT GGCACCGCCG CTCTACAGTC ATGCGGTGGC GCTGCATGCC 60
CTGGCGCTGG AGATGGCCCA ACGCGGCCAC CGGGTGACCT TTCTCACCGG CAACGTCGCC 120
TCGCTGGCAG AGCAGGAAAC GGAGCGGGTG GCGTTCTATC CACTTCCCGC CAGCGTGCAA 180
CAGGCCCAGC GCAACGTCCA GCAGCAGAGT AACGGCAACC TGCTGCGGCT GATTGCGGCC 240
ATGTCATCCC TGACCGATGT GCTCTGCCAG CAGTTGCCCG CTATTCTACA GCGGCTGGCG 300
GTGGACGCGC TGATTGTCTGA TGAGATGGAG CCCGCCGGAA GCCTGGTCGC CGAGGCGCTG 360

GGACTACCAT	TTATCTCTAT	TGCCTGCGCG	CTGCCGGTCA	ACCGCGAGCT	GCCGCTGCCG	420
GTGATGCCGT	TTCACACGC	CGAGGATAAG	AGAGCCCGTG	CGCGTTTTCA	GGTCAGCGAA	480
CGGATCTACG	ATGCGCTGAT	GTACCCGCAC	GGGCAGACGA	TCCTGCGCCA	CGCCCAGCGC	540
TTTGGTTTGC	CGGAGCGCAG	GCGTCTCGAC	GAGTGTCTCT	CGCCCCTGGC	GCAGATTAGC	600
CAGTCCGTTT	CGGCCCTCGA	CTTCCCACGC	CGGGCGCTGC	CGAACTGTTT	TACCTACGTG	660
GGAGCACTGC	GCTATCAGCC	CCCGCCGCAG	GTAGAACGCT	CGCCACGCAG	CACGCCGCGG	720
ATCTTTGCCT	CGCTGGGCAC	CCTCCAGGGC	CACCGTCTAC	GCCTGTTTCA	GAAGATCGCC	780
CGCGCCTGTG	CCAGCGTGGG	CGCGGAGGTG	ACCATTGCCC	ACTGCGATGG	CCTGACGCCC	840
GCCCAGGCCG	ACTCGCTCTA	CTGCGGCGCG	ACGGAGGTGG	TCAGCTTTGT	CGACCAGCCG	900
CGCTACGTTG	CCGAGGCTAA	TCTGGTGATC	ACCCACGGCG	GTCTCAATAC	CGTACTGGAT	960
CGGCTGGCTG	CCGCGACGCC	GGTGCTGGCG	GTGCCACTCT	CTTTCGACCA	GCCCGCCGTG	1020
GCTGCCCGGC	TGGTCTATAA	CGGGCTGGGT	CGCCGGGTAT	CGCGCTTTGC	CAGACAGCAG	1080
ACGCTGGCGG	ATGAGATTGC	CCAACCTGCT	GGGGATGAGA	CGCTGCATCA	GCGTGTGGCG	1140
ACGGCCCCGC	AGCAGCTTAA	CGACGCCGGG	GGCACGCCCC	GTTGCGGCGA	CCCTGATTGA	1200

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 59 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

Met Ala Ser Ser Val Leu Ser Ser Ala Ala Val Ala Thr Arg Ser Asn
1 5 10 15
Val Ala Gln Ala Asn Met Val Ala Pro Phe Thr Gly Leu Lys Ser Ala
20 25 30
Ala Ser Phe Pro Val Ser Arg Lys Gln Asn Leu Asp Ile Thr Ser Ile
35 40 45
Ala Ser Asn Gly Gly Arg Val Gln Cys Met Gln
50 55

-235-

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 489 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

```

Met Glu Lys Thr Val Val Ile Gly Ala Gly Phe Gly Gly Leu Ala Leu
 1              5              10              15

Ala Ile Arg Leu Gln Ala Ala Gly Ile Pro Thr Val Leu Leu Glu Gln
 20              25              30

Arg Asp Lys Pro Gly Gly Arg Ala Tyr Val Trp His Asp Gln Gly Phe
 35              40              45

Thr Phe Asp Ala Gly Pro Thr Val Ile Thr Asp Pro Thr Ala Leu Glu
 50              55              60

Ala Leu Phe Thr Leu Ala Gly Arg Arg Met Glu Asp Tyr Val Arg Leu
 65              70              75              80

Leu Pro Val Lys Pro Phe Tyr Arg Leu Cys Trp Glu Ser Gly Lys Thr
 85              90              95

Leu Asp Tyr Ala Asn Asp Ser Phe Glu Leu Glu Ala Gln Ile Thr Gln
100              105              110

Phe Asn Pro Arg Asp Val Glu Gly Tyr Arg Arg Phe Leu Ala Tyr Ser
115              120              125

Gln Ala Val Phe Gln Glu Gly Tyr Leu Arg Leu Gly Ser Val Pro Phe
130              135              140

Leu Ser Phe Arg Asp Met Leu Arg Ala Gly Pro Gln Leu Leu Lys Leu
145              150              155              160

Gln Ala Trp Gln Ser Val Tyr Gln Ser Val Ser Arg Phe Ile Glu Asp
165              170              175

Glu His Leu Arg Gln Ala Phe Ser Phe His Ser Leu Leu Val Gly Gly
180              185              190

Asn Pro Phe Thr Thr Ser Ser Ile Tyr Thr Leu Ile His Ala Leu Glu
195              200              205

Arg Glu Trp Gly Val Trp Phe Pro Glu Gly Gly Thr Gly Ala Leu Val
210              215              220

```

-236-

Asn Gly Met Val Lys Leu Phe Thr Asp Leu Gly Gly Glu Ile Glu Leu
 225 230 235 240
 Asn Ala Arg Val Glu Glu Leu Val Val Ala Asp Asn Arg Val Ser Gln
 245 250 255
 Val Arg Leu Ala Asp Gly Arg Ile Phe Asp Thr Asp Ala Val Ala Ser
 260 265 270
 Asn Ala Asp Val Val Asn Thr Tyr Lys Lys Leu Leu Gly Thr Ile Pro
 275 280 285
 Val Gly Gln Lys Arg Ala Ala Arg Leu Glu Arg Lys Ser Met Ser Asn
 290 295 300
 Ser Leu Phe Val Leu Tyr Phe Gly Leu Asn Gln Pro His Ser Gln Leu
 305 310 315 320
 Ala His His Thr Ile Cys Phe Gly Pro Arg Tyr Arg Glu Leu Ile Asp
 325 330 335
 Glu Ile Phe Thr Gly Ser Ala Leu Ala Asp Asp Phe Ser Leu Tyr Leu
 340 345 350
 His Ser Pro Cys Val Thr Asp Pro Ser Leu Ala Pro Pro Pro Cys Ala
 355 360 365
 Ser Phe Tyr Val Leu Ala Pro Val Pro His Leu Gly Asn Ala Pro Leu
 370 375 380
 Asp Trp Ala Gln Glu Gly Pro Lys Leu Arg Asp Arg Ile Phe Asp Tyr
 385 390 395 400
 Leu Glu Glu Arg Tyr Met Pro Gly Leu Arg Ser Gln Leu Val Thr Gln
 405 410 415
 Arg Ile Phe Thr Arg Gln Thr Ser Arg His Ala Trp Ile Ala Ile Leu
 420 425 430
 Gly Ser Leu Phe Ile Glu Pro Pro Ser Leu Thr Gln Gly Leu Phe Ala
 435 440 445
 Ala Asn Ala Thr Arg His Ser Asn Leu Tyr Leu Val Ala Ala Gly Thr
 450 455 460
 His Pro Gly Ala Gly Ile Pro Gly Val Val Gly Leu Ala Glu Ser Thr
 465 470 475 480
 Ala Ser Leu Met Ile Glu Asp Leu Gln
 485

-237-

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

ATAAAGACAT TGTTTTTAGA TCTGTTGTAA 30

-238-

WHAT IS CLAIMED IS:

1. An isolated DNA segment comprising a nucleotide sequence that contains at least 850 base pairs that define a structural gene for the Erwinia herbicola enzyme geranylgeranyl pyrophosphate synthase (E.C.2.5.1.29) and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.
2. The isolated DNA segment as described in claim 1 wherein said structural gene is selected from the group consisting of those present within the approximately 1030 base pair Nco I-Eco RV restriction fragment of plasmid pARC417BH having the ATCC accession No. 40755; the approximately 1000 base pair Nco I-Pvu II restriction fragment of plasmid pARC498D having the ATCC accession No. 40757, and the approximately 1150 base pair Nco I-Pvu II restriction fragment of plasmid pARC489B having ATCC accession No. 40758.
3. A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment that contains at least 850 base pairs defining a structural gene for the Erwinia herbicola enzyme geranylgeranyl pyrophosphate synthase (E.C. 2.5.1.29) and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and a promoter suitable for driving the expression of said enzyme in a compatible host organism.
4. The recombinant DNA molecule as described in claim 3 wherein said host organism is a prokaryote.
5. The recombinant DNA molecule as described in claim 3 wherein said host organism is a higher plant.

-239-

6. A method for preparing the Erwinia herbicola enzyme geranylgeranyl pyrophosphate synthase, comprising the steps of:

- 5 a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells transformed with a recombinant DNA molecule comprising an expression vector compatible with said cells operatively linked to an exogenous DNA segment containing at least 850 base pairs defining the Erwinia herbicola structural gene for geranylgeranyl
10 pyrophosphate synthase, DNA variants and analogs thereof encoding an enzyme exhibiting substantially the same biological activity; and
- 15 b. maintaining said culture for a time period sufficient for said cells to express said geranylgeranyl pyrophosphate synthase protein molecule.

7. The DNA segment as defined in claim 6 wherein said structural gene is present within the
20 approximately 1000 base pair Nco I-Pvu II restriction endonuclease fragment of plasmid pARC489D having the ATCC accession No. 40757.

8. An isolated DNA segment comprising a
25 nucleotide sequence that contains at least 927 base pairs defining an Erwinia herbicola structural gene for phytoene synthase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

30

9. The isolated DNA segment as described in claim 10 wherein said structural gene is present within the approximately 1238 base pair Pvu II-Eco RI restriction endonuclease fragment of plasmid pARC140N.

35

-240-

10. A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment that contains at least 927 base pairs defining a structural gene for the Erwinia herbicola enzyme phytoene synthase, and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and a promoter suitable for driving the expression of said enzyme in a compatible host organism.

11. The recombinant DNA molecule as described in claim 10 wherein said host organism is a prokaryote. coli.

12. The recombinant DNA molecule as described in claim 10 wherein said host organism is a higher plant.

13. A method for preparing the enzyme phytoene synthase, comprising the steps of:

a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells transformed with an exogenous recombinant DNA molecule comprising an expression vector compatible with said cells operatively linked to a DNA segment containing at least 927 base pairs defining the structural gene for Erwinia herbicola phytoene synthase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity; and

b. maintaining said culture for a time period sufficient for said cells to express said phytoene synthase protein molecule.

-241-

14. A method for producing phytoene, comprising the steps of:

5 a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells transformed with a recombinant DNA molecule containing an expression system that comprises one or more expression vectors compatible with said cells operatively linked to an exogenous DNA segment comprising (i) a nucleotide base sequence that contains
10 at least 850 base pairs defining a structural gene for Erwinia herbicola geranylgeranyl pyrophosphate synthase, and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and (ii) a nucleotide base sequence that contains at least 927 base
15 pairs defining an Erwinia herbicola structural gene for phytoene synthase, and DNA variants thereof encoding an enzyme exhibiting substantially the same biologically activity; and

20 b. maintaining said culture for a time period sufficient for said cells to express phytoene.

15. The method of preparation described in claim 14 wherein said transformed host is a prokaryote.

25 16. The method of preparation described in claim 14 wherein said transformed host is a higher plant.

30 17. A plasmid vector containing a DNA segment that encodes GGPP synthase selected from the group consisting of plasmid pARC417BH having ATCC accession number 40755, plasmid pARC489B having ATCC accession number 40758, plasmid pARC489D having ATCC accession number 40757 and plasmid pARC145G having ATCC accession
35 number 40753.

-242-

18. A plasmid vector containing a DNA segment that encodes phytoene synthase selected from the group consisting of plasmid pARC285 having ATCC accession number 40756, plasmid pARC140N having ATCC
5 accession number 40754, and plasmid pARC145G having ATCC accession number 40753.

19. An isolated DNA segment comprising a nucleotide sequence of at least 1470 base pairs that
10 defines a structural gene for the Erwinia herbicola enzyme phytoene dehydrogenase-4H and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

20. The isolated DNA segment as described in claim 19 wherein said structural gene is selected from the group consisting of those present within the approximately 1505 base pair Nco I-Nco I restriction
15 fragment of plasmid pARC496A having the ATCC accession No. 40803, the approximately 1508 base pair Sal I-Sal I restriction fragment of plasmid pARC146D having ATCC
20 Accession No. 40801, the approximately 1506 base pair Sph I-Nco I restriction fragment of plasmid pATC228 having ATCC accession No. 40802, and the approximately
25 2450 base pair Xba I-Xba I restriction fragment of plasmid pATC1616 having ATCC accession No. 40806.

21. A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment
30 that contains a nucleotide sequence of at least about 1470 base pairs defining a structural gene for the Erwinia herbicola enzyme phytoene dehydrogenase-4H and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and a

-243-

promoter suitable for driving the expression of said enzyme in a compatible host organism.

5 22. The recombinant DNA molecule as described in claim 21 wherein said host organism is a prokaryote E. coli.

10 23. The recombinant DNA molecule as described in claim 21 wherein said host organism is a higher plant.

15 24. A method for preparing the Erwinia herbicola enzyme phytoene dehydrogenase-4H, comprising the steps of:

15 a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells transformed with a recombinant DNA molecule comprising an expression vector compatible with said cells operatively linked to an exogenous DNA segment
20 containing a nucleotide sequence of at least about 1470 base pairs defining the Erwinia herbicola structural gene for phytoene dehydrogenase-4H and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity; and

25 b. maintaining said culture for a time period sufficient for said cells to express said phytoene dehydrogenase-4H protein molecule.

30 25. The method as described in claim 24 wherein said host cells are eukaryotic cells of a higher plant.

26. A method for producing lycopene comprising the steps of:

-244-

a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells that provide phytoene, and are transformed with a recombinant DNA molecule containing an expression system that
5 comprises an expression vector compatible with said host cells operatively linked to an exogenous DNA segment comprising a nucleotide base sequence of at least about 1470 base pairs defining an Erwinia herbicola structural gene for phytoene dehydrogenase-4H and DNA variants
10 thereof encoding an enzyme exhibiting substantially the same biologically activity; and

b. maintaining said culture for a time period sufficient for said cells to express phytoene dehydrogenase-4H and for said expressed phytoene
15 dehydrogenase-4H to convert the provided phytoene into lycopene.

27. The method of preparation described in claim 26 wherein said transformed host is a prokaryote.
20

28. The method of preparation described in claim 26 wherein said transformed host is a higher plant.

25 29. The method of preparation described in claim 25 wherein phytoene is provided to said host cells through an expression system comprising one or more expression vectors compatible with said host cells operatively linked to an exogenous DNA segment
30 comprising (i) a nucleotide base sequence that contains at least 850 base pairs defining a structural gene for Erwinia herbicola geranylgeranyl pyrophosphate synthase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity and (ii) a
35 nucleotide base sequence that contains at least 927 base

-245-

pairs defining an Erwinia herbicola structural gene for phytoene synthase and DNA variants encoding an enzyme thereof exhibiting substantially the same biologically activity.

5

30. The method of preparation described in claim 29 wherein said host cells are E. coli, said expression vector for phytoene dehydrogenase-4H is pARC496A having ATCC accession No. 40803, and host
10 E. coli cells are also transformed with plasmid pARC489D having ATCC accession No. 40757 and plasmid pARC140N having ATCC accession No. 40759, the expression of said plasmids pARC489D and pARC140N providing phytoene to said host cells.

15

31. The method of preparation described in claim 29 wherein said host cells are S. cerevisiae, said expression vector for phytoene dehydrogenase-4H is pARC146D having ATCC accession No. 40801, and said host
20 S. cerevisiae cells are also transformed with plasmid pARC145G having ATCC accession No. 40753, the expression of said plasmid pARC145G providing phytoene to said host cells.

25

32. The method of preparation described in claim 29 wherein said host cells are R. sphaeroides and said expression vector for phytoene dehydrogenase-4H is pATC228 having ATCC accession No. 40802.

30

33. A plasmid vector containing a DNA segment that encodes phytoene dehydrogenase-4H selected from the group consisting of plasmid pARC496A having ATCC accession No. 40803, plasmid pARC146D having ATCC accession No. 40801, plasmid pATC228 having ATCC

-246-

accession No. 40802, and plasmid pATC1616 having ATCC accession No. 40806.

5 34. An isolated DNA segment comprising a nucleotide sequence of at least about 1,125 base pairs that define a structural gene for the Erwinia herbicola enzyme lycopene cyclase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

10 35. The isolated DNA segment as described in claim 34 wherein said structural gene is selected from the group consisting of those present within the approximately 1142 base pair Sph I-Bam HI DNA fragment of sequence base pairs contained in plasmid pARC1509 having the ATCC accession number 40850 and the approximately 1156 base pair Hind III to Bam HI restriction fragment of plasmid pARC1509 having ATCC accession number 40850.

20 36. A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment that contains a nucleotide sequence of at least about 1125 base pairs defining a structural gene for the Erwinia herbicola enzyme lycopene cyclase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and a promoter suitable for driving the expression of said enzyme in a compatible host organism.

30 37. The recombinant DNA molecule as described in claim 36 wherein said host organism is a prokaryote.

-247-

38. The recombinant DNA molecule as described in claim 36 wherein said host organism is a higher plant.

5 39. A method for preparing the Erwinia herbicola enzyme lycopene cyclase, comprising the steps of:

10 a. initiating a culture, in a nutrient medium, of procaryotic or eukaryotic host cells transformed with a recombinant DNA molecule comprising an expression vector compatible with said cells operatively linked to an exogenous DNA segment containing a nucleotide sequence of at least about 1125 base pairs defining the Erwinia herbicola structural gene for lycopene cyclase and DNA variants thereof
15 encoding an enzyme exhibiting substantially the same biological activity; and

20 b. maintaining said culture for a time period sufficient for said cells to express said lycopene cyclase protein molecule.

40. A method for producing beta-carotene comprising the steps of:

25 a. initiating a culture, in a nutrient medium, of procaryotic or eukaryotic host cells that provide lycopene, and are transformed with a recombinant DNA molecule containing an expression system that comprises an expression vector compatible with said host cells operatively linked to an exogenous DNA segment
30 comprising a nucleotide base sequence of at least about 1125 base pairs defining an Erwinia herbicola structural gene for lycopene cyclase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity; and

-248-

b. maintaining said culture for a time period sufficient for said cells to express lycopene cyclase and for said expressed lycopene cyclase to convert the provided lycopene into beta-carotene.

5

41. The method of preparation described in claim 40 wherein said transformed host is a prokaryote.

42. The method of preparation described in claim 40 wherein said transformed host is a higher plant.

10

43. The method of preparation described in claim 40 including the further step of recovering said beta-carotene from said maintained culture.

15

44. The method of preparation described in claim 40 wherein lycopene is provided to said host cells through an expression system comprising one or more expression vectors compatible with said host cells operatively linked to an exogenous DNA segment comprising:

20

(i) a nucleotide base sequence that contains at least 850 base pairs defining a structural gene for Erwinia herbicola geranylgeranyl pyrophosphate synthase, and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity,

25

(ii) a nucleotide base sequence that contains at least 927 base pairs defining an Erwinia herbicola structural gene for phytoene synthase and DNA variants thereof encoding an enzyme exhibiting the same biological activity, and,

30

(iii) a nucleotide base sequence that contains at least 1470 base pairs defining an Erwinia herbicola structural gene for phytoene dehydrogenase-4H

35

-249-

and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

45. The method of preparation described in claim 44 wherein said host cells are E. coli, said expression vector for lycopene cyclase is pARC1510 having ATCC accession number 40851, and said host E. coli cells are also transformed with plasmid pARC489D having ATCC accession number 40757, plasmid pARC140N having ATCC accession number 40759, and plasmid pARC496A having ATCC accession number 40803, the expression of said plasmids pARC489D, pARC140N, and pARC496A providing lycopene to said host cells.

46. The method of preparation described in claim 44 wherein said host cells are S. cerevisiae and said expression vector for lycopene cyclase is pARC1520 having ATCC accession number 40852.

47. A plasmid vector containing a DNA segment that encodes lycopene cyclase selected from the group consisting of plasmid pARC1510 having ATCC accession number 40851, plasmid pARC1520 having ATCC accession number 40852, and plasmid pARC1509 having ATCC accession number 40850.

48. An isolated DNA segment comprising a nucleotide sequence of at least 531 base pairs that defines a structural gene for the Erwinia herbicola enzyme beta-carotene hydroxylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

49. The isolated DNA segment as described in claim 48 wherein said structural gene is present within the approximately 870 base pair Nco I-Sma I DNA fragment

-250-

of sequence base pairs contained in plasmid pARC406BH having the ATCC accession number 40945.

5 50. A recombinant DNA molecule comprising a
vector operatively linked to an exogenous DNA segment
that contains a nucleotide sequence of at least about
531 base pairs defining a structural gene for the
Erwinia herbicola enzyme beta-carotene hydroxylase and
DNA variants thereof encoding an enzyme exhibiting
10 substantially the same biological activity, and a
promoter suitable for driving the expression of said
enzyme in a compatible host organism.

15 51. The recombinant DNA molecule as described
in claim 50 wherein said host organism is a prokaryote.

20 52. The recombinant DNA molecule as described
in claim 50 wherein said host organism is a higher
plant.

25 53. A method for preparing the Erwinia
herbicola enzyme beta-carotene hydroxylase comprising
the steps of:
a. initiating a culture, in a nutrient
medium, of prokaryotic or eukaryotic host cells
transformed with a recombinant DNA molecule comprising
an expression vector compatible with said cells
operatively linked to an exogenous DNA segment
containing a nucleotide sequence of at least 531 base
30 pairs defining the Erwinia herbicola structural gene for
beta-carotene hydroxylase and DNA variants thereof
encoding an enzyme exhibiting substantially the same
biological activity; and

-251-

b. maintaining said culture for a time period sufficient for said cells to express said beta-carotene hydroxylase protein molecule.

5 54. A method for producing zeaxanthin comprising the steps of:

 a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells that provide beta-carotene and are transformed with a
10 recombinant DNA molecule containing an expression system that comprises an expression vector compatible with said host cells operatively linked to an exogenous DNA segment comprising a nucleotide base sequence of at least 531 base pairs defining an Erwinia herbicola
15 structural gene for beta-carotene hydroxylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity; and

 b. maintaining said culture for a time period sufficient for said cells to express beta-carotene hydroxylase and for said expressed beta-carotene hydroxylase to convert the provided beta-carotene into zeaxanthin.
20

25 55. The method of preparation described in claim 54 wherein said transformed host is a prokaryote.

 56. The method of preparation described in claim 54 wherein said transformed host is a higher plant.
30

 57. The method of preparation described in claim 54 wherein beta-carotene is provided to said host cells through an expression system comprising one or more expression vectors compatible with said host cells

-252-

operatively linked to an exogenous DNA segment comprising:

- 5 (i) a nucleotide base sequence that contains at least 850 base pairs defining a structural gene for Erwinia herbicola geranylgeranyl pyrophosphate synthase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity,
- 10 (ii) a nucleotide base sequence that contains at least 927 base pairs defining an Erwinia herbicola structural gene for phytoene synthase and DNA variants thereof encoding an enzyme exhibiting the same biological activity,
- 15 (iii) a nucleotide base sequence that contains at least 1470 base pairs defining an Erwinia herbicola structural gene for phytoene dehydrogenase-4H and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and
- 20 (iv) a nucleotide base sequence that contains at least 1,125 base pairs defining an Erwinia structural gene for lycopene cyclase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

25 58. The method of preparation described in claim 57 wherein said host cells are E. coli, said expression vector for beta-carotene hydroxylase is pARC406BH having ATCC accession number 40946, and said host E. coli cells are also transformed with plasmid pARC489D having ATCC accession number 40757, plasmid
30 pARC140N having ATCC accession number 40759, plasmid pARC496A having ATCC accession number 40803 and plasmid pARC1509 having ATCC accession number 40850, the expression of said plasmids pARC489D, pARC140N, pARC496A and pARC1509 providing beta-carotene to said host cells.

35

-253-

59. The method of preparation described in claim 57 wherein said host cells are S. cerevisiae, said expression vector for beta-carotene hydroxylase is pARC145H having ATCC accession number 40944.

5

60. A plasmid vector containing a DNA segment that encodes beta-carotene hydroxylase selected from the group consisting of plasmid pARC406BH having ATCC accession number 40945, plasmid pARC145H having ATCC accession number 40944, and plasmid pARC404BH having ATCC accession number 40943.

10

61. An isolated DNA segment comprising a nucleotide sequence of at least 1200 base pairs that defines a structural gene for the Erwinia herbicola enzyme zeaxanthin glycosylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

15

62. The isolated DNA segment as described in claim 61 wherein said structural gene is present within the approximately 1390 base pair Nde I-Ava I DNA fragment of sequence base pairs contained in plasmid pARC2019 having the ATCC accession number 40974.

20

25

63. A recombinant DNA molecule comprising a vector operatively linked to an exogenous DNA segment that contains a nucleotide sequence of at least about 1200 base pairs defining a structural gene for the Erwinia herbicola enzyme zeaxanthin glycosylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and a promoter suitable for driving the expression of said enzyme in a compatible host organism.

30

35

-254-

64. The recombinant DNA molecule as described in claim 63 wherein said host organism is a prokaryote.

5 65. The recombinant DNA molecule as described in claim 63 wherein said host organism is a higher plant.

10 66. A method for preparing the Erwinia herbicola enzyme zeaxanthin glycosylase comprising the steps of:

a. initiating a culture, in a nutrient medium, of procaryotic or eukaryotic host cells transformed with a recombinant DNA molecule comprising an expression vector compatible with said cells
15 operatively linked to an exogenous DNA segment containing a nucleotide sequence of at least 1200 base pairs defining the Erwinia herbicola structural gene for zeaxanthin glycosylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological
20 activity; and

b. maintaining said culture for a time period sufficient for said cells to express said zeaxanthin glycosylase protein molecule.

25 67. A method for producing zeaxanthin diglucoside comprising the steps of:

a. initiating a culture, in a nutrient medium, of prokaryotic or eukaryotic host cells that provide zeaxanthin and are transformed with a
30 recombinant DNA molecule containing an expression system that comprises an expression vector compatible with said host cells operatively linked to an exogenous DNA segment comprising a nucleotide base sequence of at least 1200 base pairs defining an Erwinia herbicola
35 structural gene for zeaxanthin glycosylase and DNA

-255-

variants thereof encoding an enzyme exhibiting substantially the same biological activity;

b. maintaining said culture for a time period sufficient for said cells to express zeaxanthin glycosylase and for said expressed zeaxanthin glycosylase to convert the provided zeaxanthin into zeaxanthin diglucoside; and

c. recovering said zeaxanthin diglucoside.

68. The method of preparation described in claim 67 wherein said transformed host is a prokaryote.

69. The method of preparation described in claim 67 wherein said transformed host is a higher plant.

70. The method of preparation described in claim 67 wherein zeaxanthin is provided to said host cells through an expression system comprising one or more expression vectors compatible with said host cells operatively linked to an exogenous DNA segment comprising:

(i) a nucleotide base sequence that contains at least 850 base pairs defining a structural gene for Erwinia herbicola geranylgeranyl pyrophosphate synthase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity,

(ii) a nucleotide base sequence that contains at least 927 base pairs defining an Erwinia herbicola structural gene for phytoene synthase and DNA variants thereof encoding an enzyme exhibiting the same biological activity,

(iii) a nucleotide base sequence that contains at least 1470 base pairs defining an Erwinia

-256-

herbicola structural gene for phytoene dehydrogenase-4H and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity,

5 (iv) a nucleotide base sequence that contains at least 1125 base pairs defining an Erwinia herbicola structural gene for lycopene cyclase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity, and

10 (v) a nucleotide base sequence that contains at least 531 base pairs defining an Erwinia herbicola structural gene for beta-carotene hydroxylase and DNA variants thereof encoding an enzyme exhibiting substantially the same biological activity.

15 71. The method of preparation described in claim 70 wherein said host cells are E. coli, said expression vector for zeaxanthin glycosylase is pARC2019 having ATCC accession number 40974, and said host E. coli cells are transformed with plasmid pARC489D
20 having ATCC accession number 40757, plasmid pARC140N having ATCC accession number 40759, plasmid pARC496A having ATCC accession number 40803, plasmid pARC1509 having ATCC accession number 40850 and plasmid pARC406BH having ATCC accession number 40946, the expression of
25 said plasmids pARC489D, pARC140N, pARC496A, pARC1509 and pARC406BH providing zeaxanthin to said host cells.

30 72. The method of preparation described in claim 70 wherein said host cells are S. cerevisiae.

73. A plasmid vector containing a DNA segment that encodes zeaxanthin glycosylase that is plasmid pARC2019 having ATCC accession number 40974.

-257-

74. A method for protecting a higher plant from the herbicide norflurazon that comprises the steps of:

- 5 a. transforming a higher plant to be protected with a recombinant DNA molecule that encodes a structural gene for the Erwinia herbicola enzyme phytoene dehydrogenase-4H or a DNA variant thereof that encode enzyme exhibiting substantially the same biological activity;
- 10 b. maintaining the transformed plant for a time period sufficient for said phytoene dehydrogenase-4H to be expressed; and
- 15 c. treating the transformed plant with a herbicidal amount of norflurazon.

75. The method as described in claim 74 wherein said recombinant DNA molecule includes an about 177 base pair sequence that encodes a chloroplast transit peptide of tobacco ribulose bis-phosphate carboxylase-oxygenase operatively linked in frame to the 5' end phytoene dehydrogenase-4H structural gene.

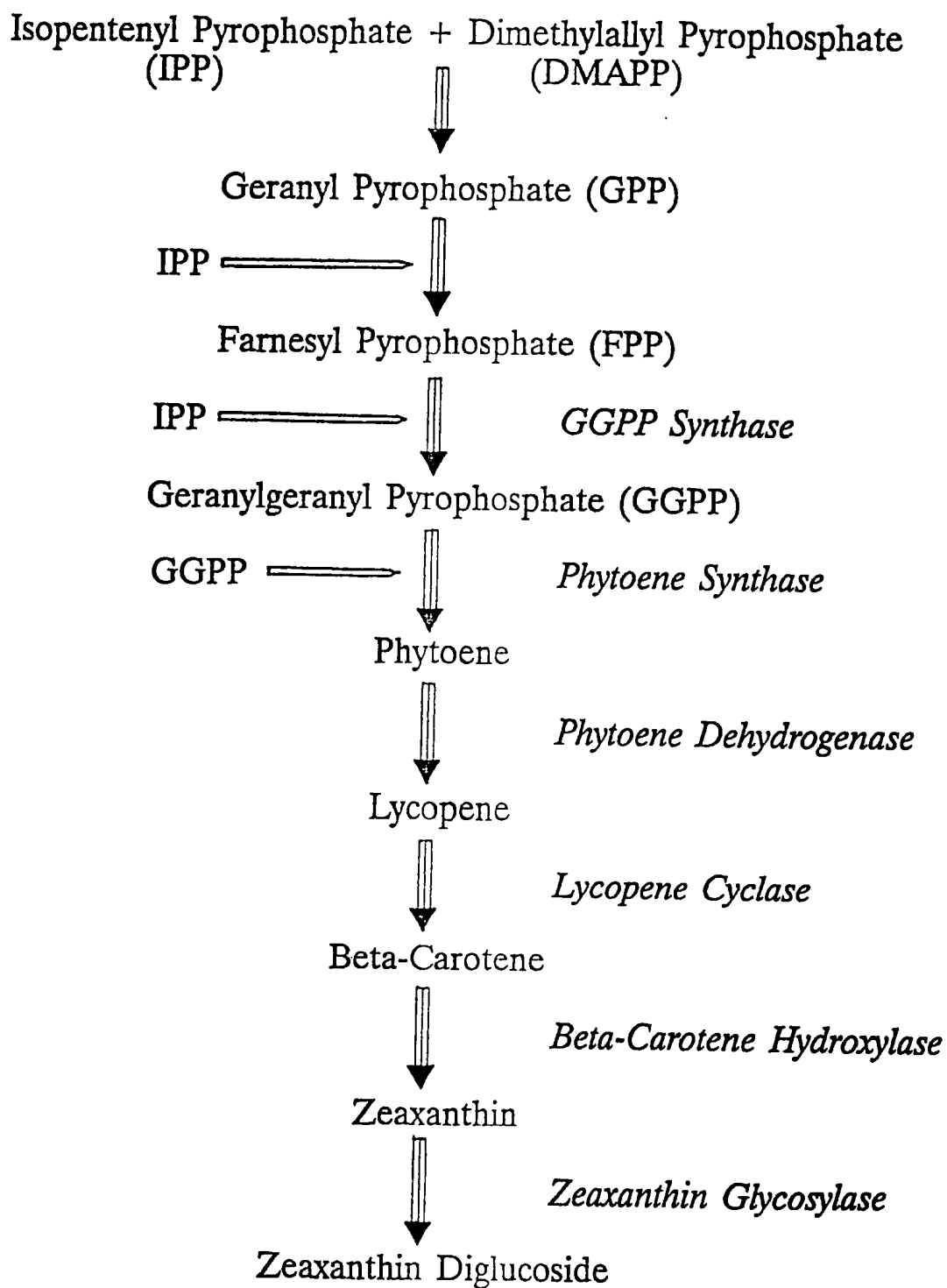
20

76. The method as described in claim 75 wherein said recombinant DNA molecule includes the approximately 2450 base pair Xba I-Xba I fragment present in plasmid pATC1616 having ATCC accession number 40806.

25

Figure 1

Carotenoid Biosynthesis Scheme



Bgl II
 AGATCTAAAGGCACAGCGTCTCATGCTTCGCACAATGTAAACTGCTTCAGAACCTGGCG 60

 Hpa I
 AGAGCTATCCGGCGGCTACGGTTAACTGATACTAAAGACAATTCAGCGGGTAACCTT 120

 Nru I
 GCAATGGTGAGTGGCAGTAAAGCGGGCGTTTCGCCCTCATCGCGAAATAGAGGTAATGAGA 180
 MetValSerGlySerLysAlaGlyValSerProHisArgGluIleGluValMetArg

 CAATCCATTGACGATCACCTGGCTGGCCTGTACCTGAACCGACAGCCAGGATATCGTC 240
 GlnSerIleAspAspHisLeuAlaGlyLeuLeuProGluThrAspSerGlnAspIleVal

 Bam HI
 AGCCTTGCGATGCGTGAAGCGGTCATGGCACCCGGTAAACGGATCCGTCGCTGCTGATG 300
 SerLeuAlaMetArgGluGlyValMetAlaProGlyLysArgIleArgProLeuLeuMet

 CTGCTGGCCCGCCGACCTCCGCTACCAGGGCAGTATGCCCTACGCTGCTCGATCTCGCC 360
 LeuLeuAlaAlaArgAspLeuArgTyrGlnGlySerMetProThrLeuLeuAspLeuAla

 TCGCGCCGTTGAACCCATACCGCGTCGCTGATGCTCGACGACATGCCCTGCATGGAC 420
 CysAlaValGluLeuThrHisThrAlaSerLeuMetLeuAspAspMetProCysMetAsp

FIGURE 2-1

3

480
ACCGCCGAGCTGCGCCGGTCAAGCCACTACCCACAAAAATTGGTGAGAGCGTGCGG
AsnAlaGluLeuArgArgGlyGlnProThrHisLysLysPheGlyGluSerValAla

540
ATCCTTGCCCTCCGTTGGGCTGCTCTCTAAAGCCTTTGGTCTGATCGCCGCCACCGCGGAT
IleLeuAlaSerValGlyLeuLeuSerLysAlaPheGlyLeuIleAlaAlaThrGlyAsp

600
CTGCCGGGGAGAGCGTGCCAGGCGGTCAACGAGCTCTACCGCCGTGGGGCTGCAG
LeuProGlyGluArgAlaGlnAlaValAsnGluLeuSerThrAlaValGlyLeuGln

660
GGCCTGGTACTGGGGCAGTTTCGGGATCTTAACGATGCCGCCCTCGACCGTACCCCTGAC
GlyLeuValLeuGlyGlnPheArgAspLeuAsnAspAlaAlaLeuAspArgThrProAsp

720
GCTATCCTCAGCACCAACCACTCAAGACCGGCATTCTGTTACGCGGATGCTGCAGATC
AlaIleLeuSerThrAsnHisLeuLysThrGlyIleLeuPheSerAlaMetLeuGlnIle

780
GTCGCCATTGCTCCGCCCTCGTCGCCGAGCACGCGAGAGACGCTGCACGCCCTTCGCCCTC
ValAlaIleAlaSerAlaSerProSerThrArgGluThrLeuHisAlaPheAlaLeu

840
GACTTCGGCCAGGCGTTTCAACTGCTGGACGATCTGCGTGACGATCACCCGGAACCGGT
AspPheGlyGlnAlaPheGlnLeuLeuAspLeuArgAspHisProGluThrGly

FIGURE 2-2

AAAGATCGCAATAAGGACGCGGGAATAATCGACGCTGGTCAACCGGCTGGCGCAGACGCG 900
LysAspArgAsnLysAspAlaGlyLysSerThrLeuValAsnArgLeuGlyAlaAspAla

GCCCGGCAAAAGCTGCGGAGCATATTGATTCCGCCGACAAACACCTCACTTTTGCCCTGT 960
AlaArgGlnLysLeuArgGluHisIleAspSerAlaAspLysHisLeuThrPheAlaCys

Bal I

CCGCAGGGCGGCCATCCGACAGTTTATGCATCTGTGGTTTGGCCATCACCTTGCCGAC 1020
ProGlnGlyGlyAlaIleArgGlnPheMetHisLeuTrpPheGlyHisHisLeuAlaAsp

TGGTCACCGGTCAAGAAATCGCCCTGATACCGCCCTTTTGGGTTCAAGCAGTACATAACG 1080
TrpSerProValMetLysIleAla

ATGGAACCAATTACAGGAGTAGTGATGAATGAAGACGAGCGCCCTTGTTCAGCGTAAGA 1140

Eco RV
ACGATCATCTGGATATC

FIGURE 2-3

Bgl II
 AGATCTAAAGGCACAGCGTCTCATGCTTCGCACAATGTAAACTGCTTCAGAACCTGGCG 60

 Hpa I
 AGAGCTATCCGCGGCTCTACGGTTAACTGATACTAAAGACAATTCAGCGGGTAACCTT 120

 Nco I Nru I
 GCAATGGTGAGTGCCAGTAAAGCGGGCGTCATGCGCGAATTCGAAATAGAGGTAATGAGA 180
 MetAlaGluPheGluIleGluValMetArg

 CAATCCATTGACGATCACCTGGCTGGCCTGTACCTGAAACCGACAGCCAGGATATCGTC 240
 GlnSerIleAspAspHisLeuAlaGlyLeuLeuProGluThrAspSerGlnAspIleVal

 Bam HI
 AGCCTTGGCGATGCGTGAAAGCGGTCATGGCACCCGGTAAACGGATCCGTCGCTGCTGATG 300
 SerLeuAlaMetArgGluGlyValMetAlaProGlyLysArgIleArgProLeuLeuMet

 CTGCTGGCCCGCCGACCTCCGCTACCAGGCAGTATGCCCTACGCTGCTCGATCTCGCC 360
 LeuLeuAlaAlaArgAspLeuArgTyrGlnGlySerMetProThrLeuLeuAspLeuAla

 TGGCCCGTTGAACCTACCCATACCGCGTCGCTGATGCTCGACGACATGCCCTGCATGGAC 420
 CysAlaValGluLeuThrHisThrAlaSerLeuMetLeuAspAspMetProCysMetAsp

FIGURE 3-1

ACCGCCGAGCTGCGCCGGTCCAGCCCACTACCCACAAATAATTGGTGAGAGCGTGCGG 480
AsnAlaGluLeuArgArgGlyGlnProThrThrHisLysLysPheGlyGluSerValAla

ATCCTTGCCCTCCGTTGGCTGCTCTCTAAAGCCTTTGGTCTGATCGCCGCCACCGCGGAT 540
IleLeuAlaSerValGlyLeuLeuSerLysAlaPheGlyLeuIleAlaAlaThrGlyAsp

CTGCCGGGGAGAGCGTGCCCGGGTCAACGAGCTCTCTACCGCCGTGGGGCTGCAG 600
LeuProGlyGluArgArgAlaGlnAlaValAsnGluLeuSerThrAlaValGlyLeuGln

GGCCTGGTACTGGGGCAGTTTCGCGATCTTAACGATGCCGCCCTCGACCGTACCCCTGAC 660
GlyLeuValLeuGlyGlnPheArgAspLeuAsnAspAlaAlaLeuAspArgThrProAsp

GCTATCCTCAGCACCAACCACCTCAAGACCGGCATTCTGTTCAGCGCGATGCTGCAGATC 720
AlaIleLeuSerThrAsnHisLeuLysThrGlyIleLeuPheSerAlaMetLeuGlnIle

GTCCGCAATTGCTTCGCTCGTCCGAGCACGCGAGAGACGCTGCACGCCTTCGCCCTC 780
ValAlaIleAlaSerAlaSerProSerThrArgGluThrLeuHisAlaPheAlaLeu

GACTTCGGCCAGCGGTTCAACTGCTGGACGATCTGCGTGACGATCACCCGGAACCGGT 840
AspPheGlyGlnAlaPheGlnLeuLeuAspLeuArgAspAspHisProGluThrGly

FIGURE 3-2

7

AAAGATCGCAATAAGGACGCGGGAATAATCGACGCTGGTCAACCGGCTGGGCGCAGACGCG 900
LysAspArgAsnLysAspAlaGlyLysSerThrLeuValAsnArgLeuGlyAlaAspAla

GCCCGGCAAAAGCTGCGGAGCATATTGATTCGCCGACAAACACCTCACTTTGCCTGT 960
AlaArgGlnLysLeuArgGluHisIleAspSerAlaAspLysHisLeuThrPheAlaCys

CCGCAGGCGCGCCATCCGACAGTTTATGCACTGTGGTTTGGCCATCACCTTGCCGAC 1020
ProGlnGlyGlyAlaIleArgGlnPheMetHisLeuTrpPheGlyHisHisLeuAlaAsp

TGGTCACCGGTCAAGAAATCGCCCTGATACCGCCCTTTTGGGTTCAAGCAGTACATAACG 1080
TrpSerProValMetLysIleAla

ATGGAACCATTAACAGGAGTAGTGATGAATGAAGACGAGCGCCTTGTTTCAGCGTAAGA 1140
Eco RV
ACGATCATCTGGATATC

FIGURE 3-3

480 TATCACGTGGCGCGGTGGTGGTCTGATGATGGCCAGGCGTGGCGGTGGCGGATGAG
TyrHisValAlaGlyValValGlyLeuMetMetAlaArgValMetGlyValArgAspGlu

540 CGGGTGCTGGATCGCGCCTGCGATCTGGGGCTGGCCTTCCAGCTGACGAATATGGCCCGG
Sma I
ArgValLeuAspArgAlaCysAspLeuGlyLeuAlaPheGlnLeuThrAsnMetAlaArg

600 GATATTATTGACGATCGCGGCTATTGACCGCTGCTATCTGCCCGCCGAGTGGCTGCAGGAT
Pst I
AspIleIleAspAspAlaIleAlaIleAspArgCysTyrLeuProAlaGluTrpLeuGlnAsp

660 GCCGGGTGGCCCCGGAGAACTATGCCGCGGGGAGAAATCGCCCCGCGTGGCGCGGTGG
AlaGlyLeuAlaProGluAsnTyrAlaAlaArgGluAsnArgProAlaLeuAlaArgTrp

720 CGGAGGCTTATTGATGCCGCGAGAGCCGTACTACATCTCTCCAGCGCCGGCTACACGAT
ArgArgLeuIleAspAlaAlaGluProTyrTyrIleSerSerGlnAlaGlyLeuHisAsp

780 CTCGGCGCGGCTCCGCGTGGGCGATCGCCACCGCCCGCAGCGTCTACCGGAGATCGGT
LeuArgArgArgSerAlaTrpAlaIleAlaThrAlaArgSerValTyrArgGluIleGly

840 ATTAAGGTAAAGCGCGGAGGCAGCGCCTGGGATCGCCCGCCAGCACACCAAGGT
IleLysValLysAlaIleGlyGlySerAlaTrpAspArgArgGlnHisThrSerLysGly

FIGURE 4-2

GAATAATTGCCATGCTGATGGCGGCACCGGGCAGGTTATTCGGGCGAAGACGAGG 900
GluLysIleAlaMetLeuMetAlaAlaProGlyGlnValIleArgAlaLysThrThrArg

GTGACGCCCGCTCCGGCCGGTCTTTGGCAGCGTCCCGTTAGCGGGCGGCCATGACGTT 960
ValThrProArgProAlaGlyLeuTrpGlnArgProVal

CACGCAGGATCGCCTGTAGGTCGGCAGGCTTGCGGGCGTAATAAACC GAAGAGACGC 1020

AGCCCTCCCGCGCGCACCGCGTGGTGCGAGCGGTGGCGGACGTAGAGCCGCTTCAGGT 1080

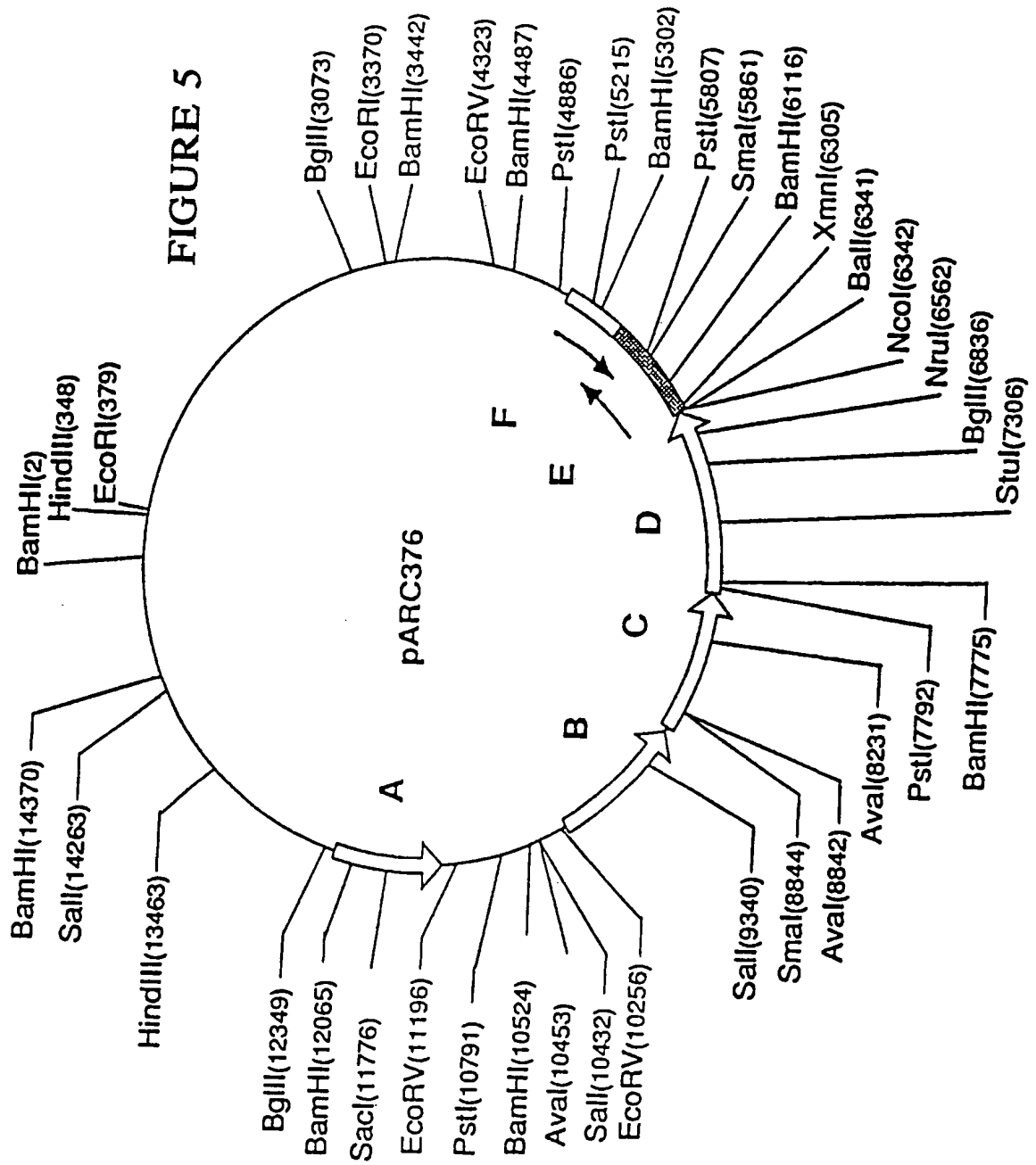
Bam HI

AGCCCGCGCGCGGATCCAGTGGAAGGCCAGCGCTGATGCACCAGACCGTCGTGCACCA 1140

Pst I

GGAAGTAGACAGGCCATAGACCGTCAATGCCCGCAGCCCAATCCACTGCAGGGGCCAAAC 1200

FIGURE 4-3



12

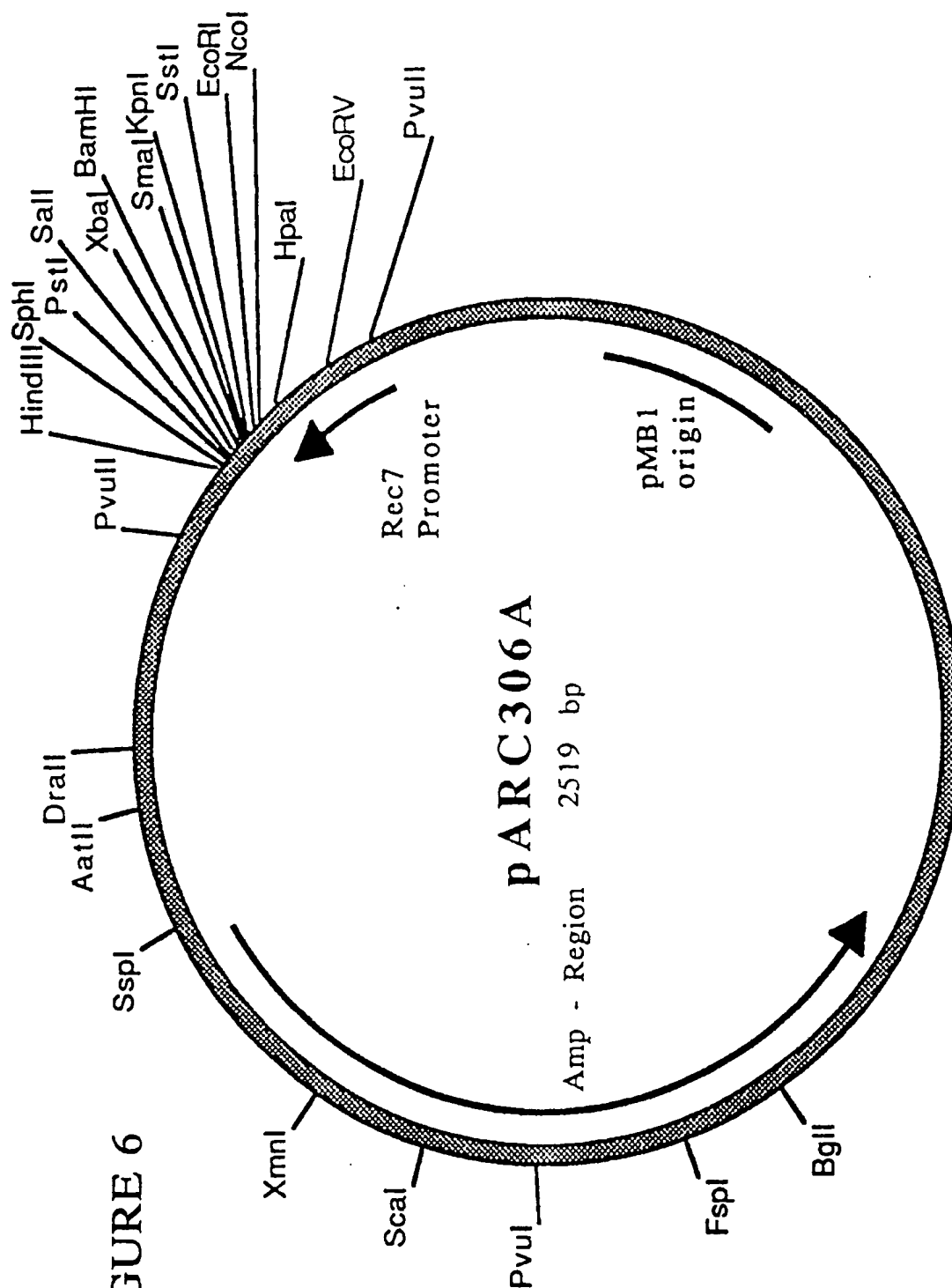
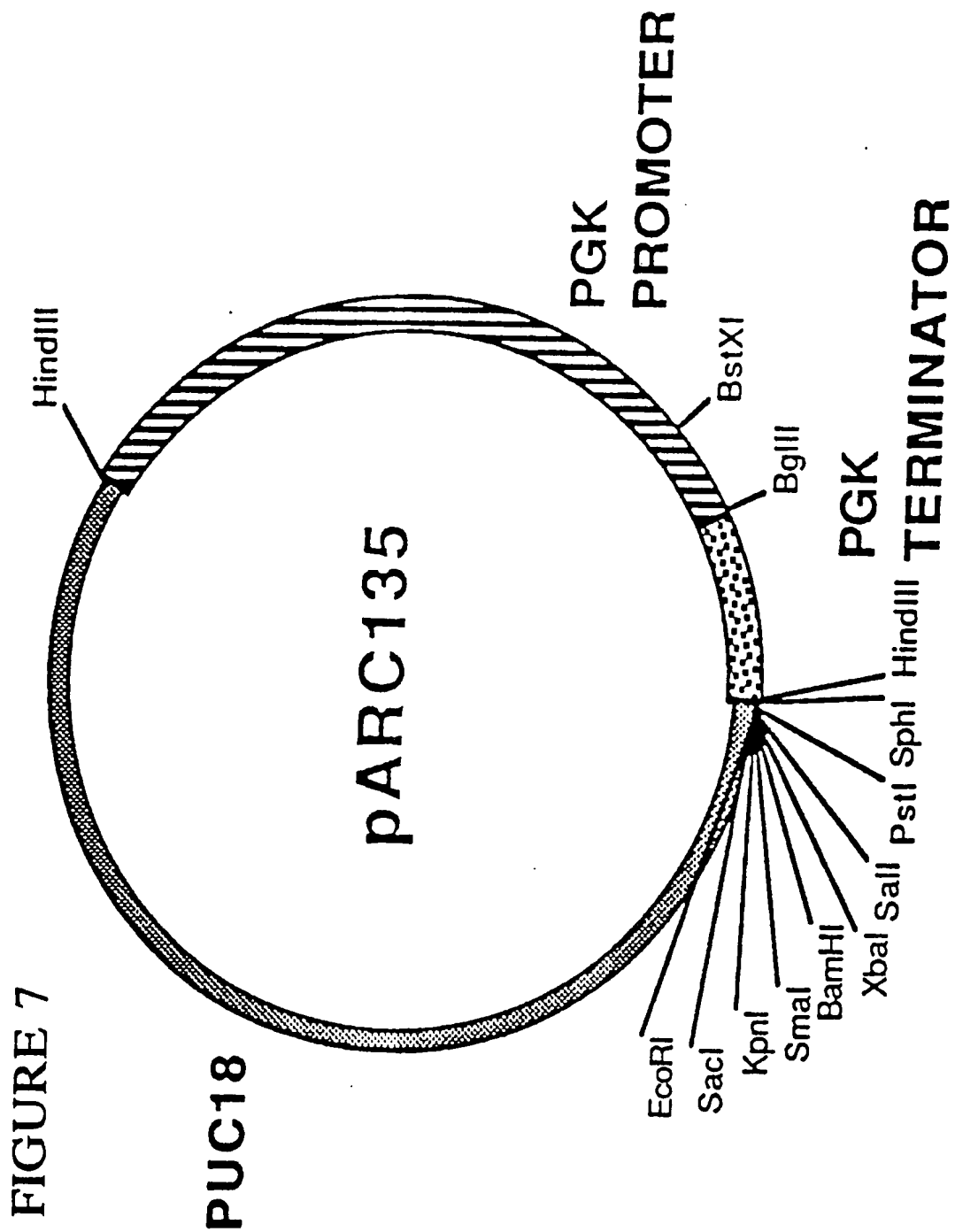


FIGURE 6

pARC306A

2519 bp



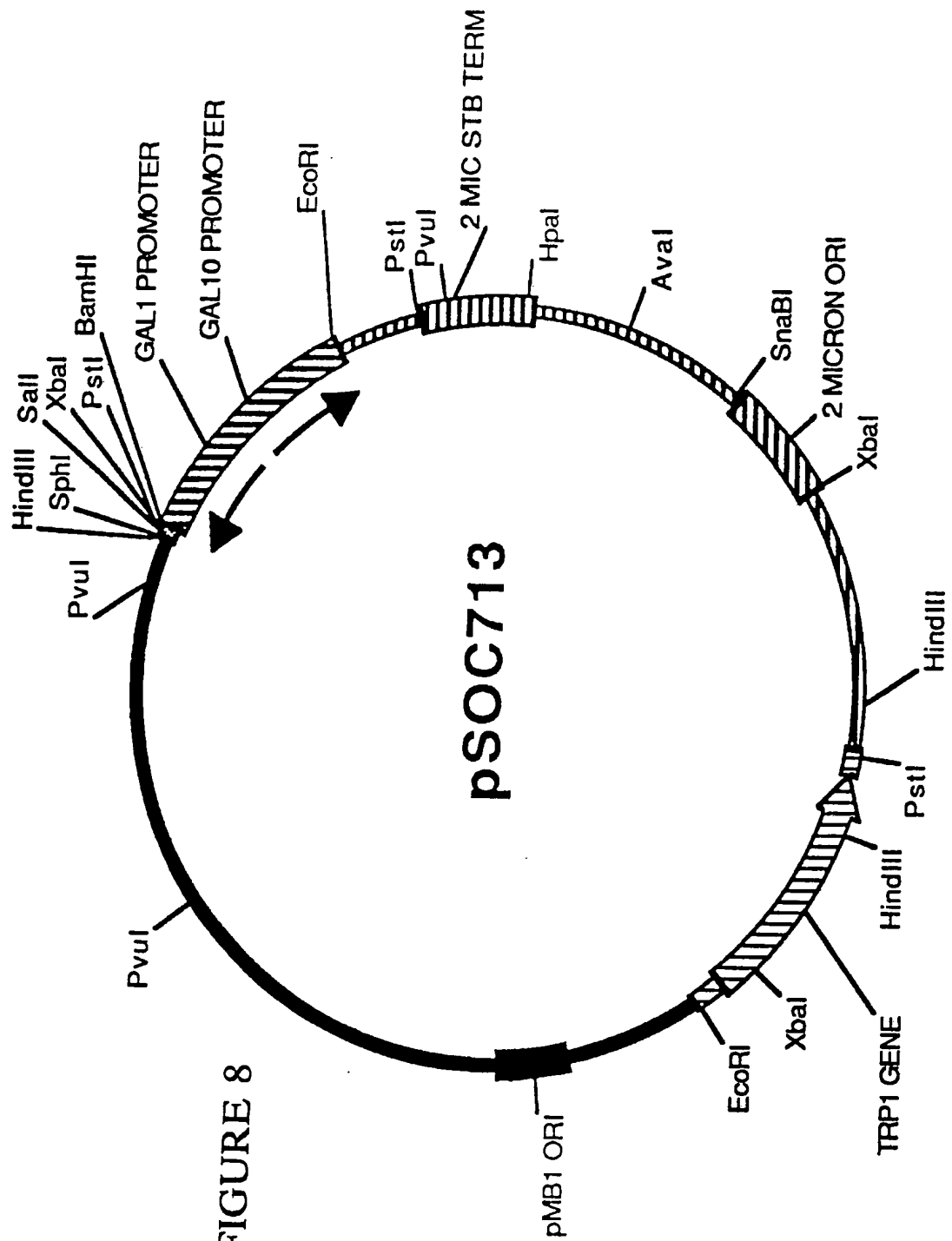


FIGURE 8

15

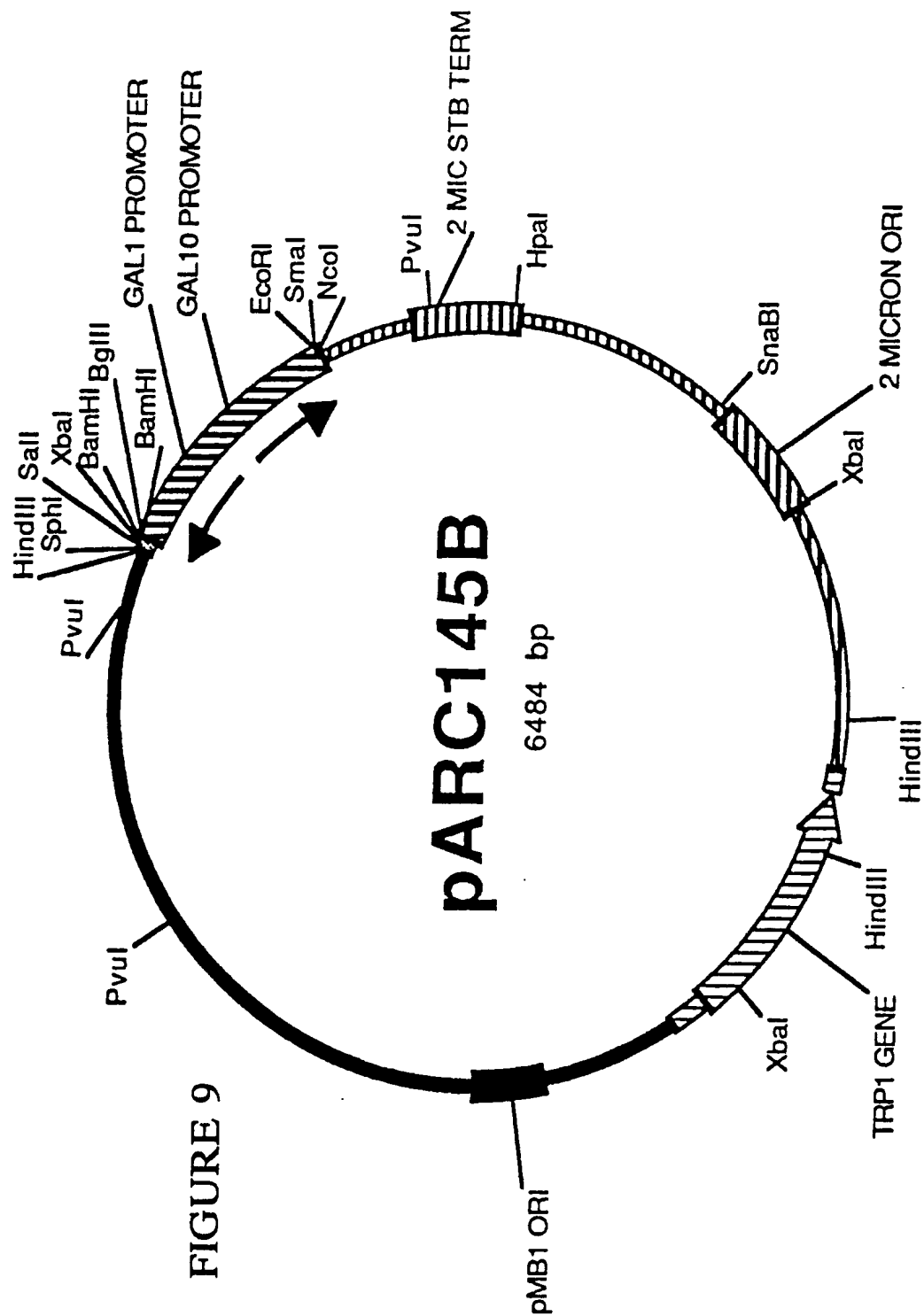


FIGURE 9

16

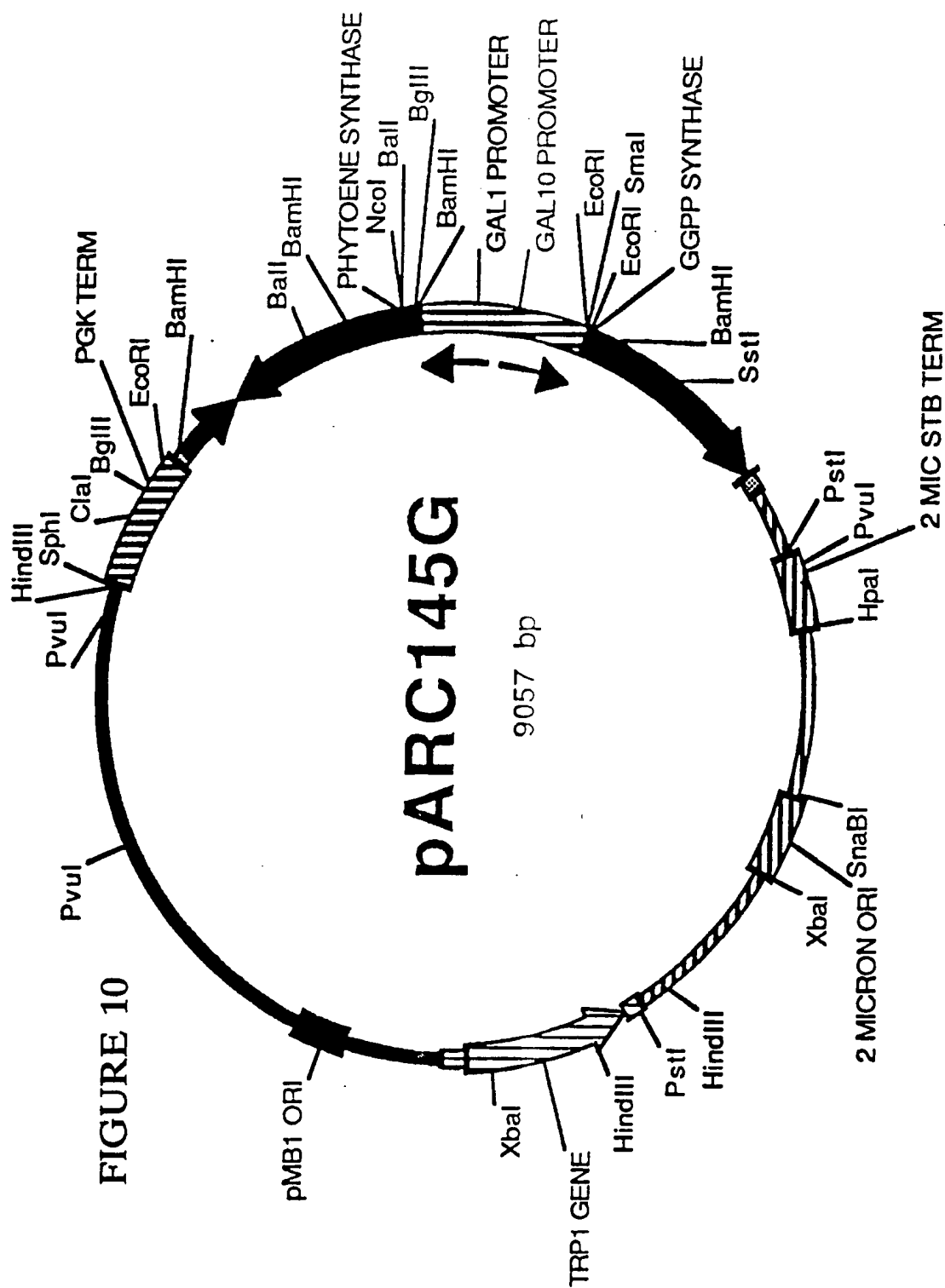


FIGURE 11-1

Nco I

TAAACCATGGAAAAACCGTTGTGATTGGCGCAGGCTTTGGTGGCCTGGCGCTGGCGATT
MetLysLysThrValValIleGlyAlaGlyPheGlyGlyLeuAlaLeuAlaIle 60

Pst I Bam HI

CGCCTGCAGGCGGCAGGATCCCAACCGTACTGCTGGAGCAGCGGACAAGCCCGCGGT
ArgLeuGlnAlaAlaGlyIleProThrValLeuLeuGluGlnArgAspLysProGlyGly 120

CGGGCCTACGTCTGGCATGACCAGGGCTTTACCTTTGACGCCGGCCGACGGTGATCACC
ArgAlaTyrValTrpHisAspGlnGlyPheThrPheAspAlaGlyProThrValIleThr 180

GATCCTACCGCGCTTGAGGCGCTGTTCACCTGGCCGGCAGCGCATGGAGGATTACGTC
AspProThrAlaLeuGluAlaLeuPheThrLeuAlaGlyArgArgMetGluAspTyrVal 240

AGGCTGCTGCCGGTAAACCCCTTCTACCGACTCTGCTGGAGTCCGGGAAGACCCCTCGAC
ArgLeuLeuProValLysProPheTyrArgLeuCysTrpGluSerGlyLysThrLeuAsp 300

TATGCTAACGACAGCTTCGAGCTTGAGGCGCAGATTACCCAGTTCAACCCCGGACGTC
TyrAlaAsnAspSerPheGluLeuGluAlaGlnIleThrGlnPheAsnProArgAspVal 360

GAGGGCTACCGCGCTTTCTGGCTTACTCCCAGCGGTATTCAGGAGGGATATTGCGC
GluGlyTyrArgArgPheLeuAlaTyrSerGlnAlaValPheGlnGluGlyTyrLeuArg 420

FIGURE 11-2

Nru I

CTCGGCAGCGTGCCGTTCCCTCTCTTTTCGCGACATGCTGCGCGCGCGCGCAGCTGCTT 480
LeuGlySerValProPheLeuSerPheArgAspMetLeuArgAlaGlyProGlnLeuLeu

AAGCTCCAGCGTGCGCAGAGCGTCTACCACTCGGTTTCGCGCTTTATTGAGGATGAGCAT 540
LysLeuGlnAlaTrpGlnSerValTyrGlnSerValSerArgPheIleGluAspGluHis

CTGCGGCAGGCCCTTCTCGTTCCACTCCCTGCTGGTAGCGCGCAACCCCTTCACCACTCG 600
LeuArgGlnAlaPheSerPheHisSerLeuLeuValGlyGlyAsnProPheThrThrSer

TCCATCTACACCCCTGATCCACGCCCTTGAGCGGGAGTGGGGGCTGGTTCCTGAGGGC 660
SerIleTyrThrLeuIleHisAlaLeuGluArgGluTrpGlyValTrpPheProGluGly

GGCACCGGGCGCTGGTGAACGGCATGGTGAAAGCTGTTTACCGATCTGGGCGGGAGATC 720
GlyThrGlyAlaLeuValAsnGlyMetValLysLeuPheThrAspLeuGlyGlyGluIle

Sma I

GAACTCAACGCCCGGTCGAAGAGCTGGTGGTGGCCGATAACCGCGTAAGCCAGGTCCGG 780
GluLeuAsnAlaArgValGluGluLeuValAlaAlaAspAsnArgValSerGlnValArg

CTCGCGGATGGTCGGATCTTTGACACCGACGCCGTAGCCTCGAACGCTGACGTGTGAAC 840
LeuAlaAspGlyArgIlePheAspThrAspAlaValAlaSerAsnAlaAspValValAsn

FIGURE 11-3

ACCTATAAAAGCTGCTCGGCACCATACCGTGGGGCAGAAAGCGGCGGCACGGCTGGAG ThrTyrLysLysLeuLeuGlyThrIleProValGlyGlnLysArgAlaAlaArgLeuGlu	900
CGCAAGAGCATGAGCAACTCGCTGTTGTGCTCTACTTCGGCCTGAACAGCCTCATTTCC ArgLysSerMetSerAsnSerLeuPheValLeuTyrPheGlyLeuAsnGlnProHisSer	960
CAGCTGGCGCACCATCTGTTTTGGTCCCCGCTACCGGAGCTGATCGACGAGATC GlnLeuAlaHisThrIleCysPheGlyProArgTyrArgGluLeuIleAspGluIle	1020
Bgl II	
TTTACCGGCAGCGCGCTGGCGGATGACTTCTCGCTCTACCTGCACCTGCCCTGCGTGACC PheThrGlySerAlaLeuAlaAspPheSerLeuTyrLeuHisSerProCysValThr	1080
GATCCCTCGCTCGCGCCTCCCCGTCGCCAGCTTCTACGTGCTGGCCCCGGTGCCGCAT AspProSerLeuAlaProProProCysAlaSerPheTyrValLeuAlaProValProHis	1140
CTTGGAACGCGCGCTGGACTGGGCGCAGGAGGGCCGAAGCTGCGCGACCGCATCTTT LeuGlyAsnAlaProLeuAspTrpAlaGlnGlyProLysLeuArgAspArgIlePhe	1200
GACTACCTTGAAGAGCGCTATATGCCCGGCTGCGTAGCCAGCTGGTGACCCAGCGGATC AspTyrLeuGluGluArgTyrMetProGlyLeuArgSerGlnLeuValThrGlnArgIle	1260

20

TTTACCCGGCAGACTTCACGACACGCTTGGATCGCGATCTTGGATCGCTTTTCATCGAG 1320
PheThrArgGlnThrSerArgHisAlaTrpIleAlaIleLeuGlySerLeuPheIleGlu

CCGCCCTTCGTTGACCCAGGCTTGTTCCGCCGCAACGCGACACGACATTCAAACCTCTAC 1380
ProProSerLeuThrGlnGlyLeuPheAlaAlaAsnAlaThrArgHisSerAsnLeuTyr

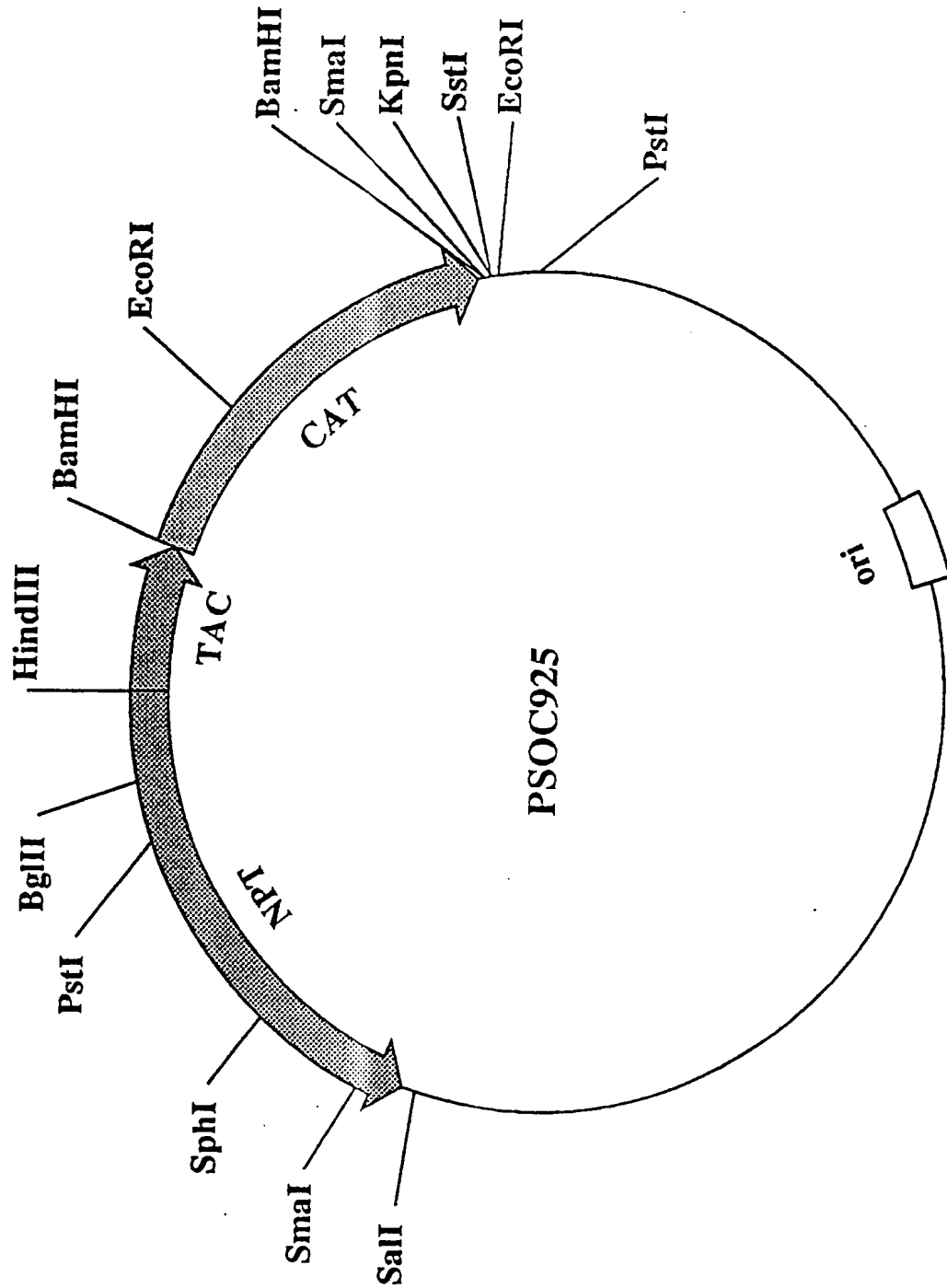
CTGGTGGCCCGCAGGTACTCACCCCTGGCGCGGCATTCTCTGGCGTAGTGGGCCCTCGCCGAA 1440
LeuValAlaAlaGlyThrHisProGlyAlaGlyIleProGlyValValGlyLeuAlaGlu

AGCACCCGCGCCTGATGATTGAGGATCTGCAATGAGCCAACCGCCGCTGCTTGACCACG 1500
SerThrAlaSerLeuMetIleGluAspLeuGln

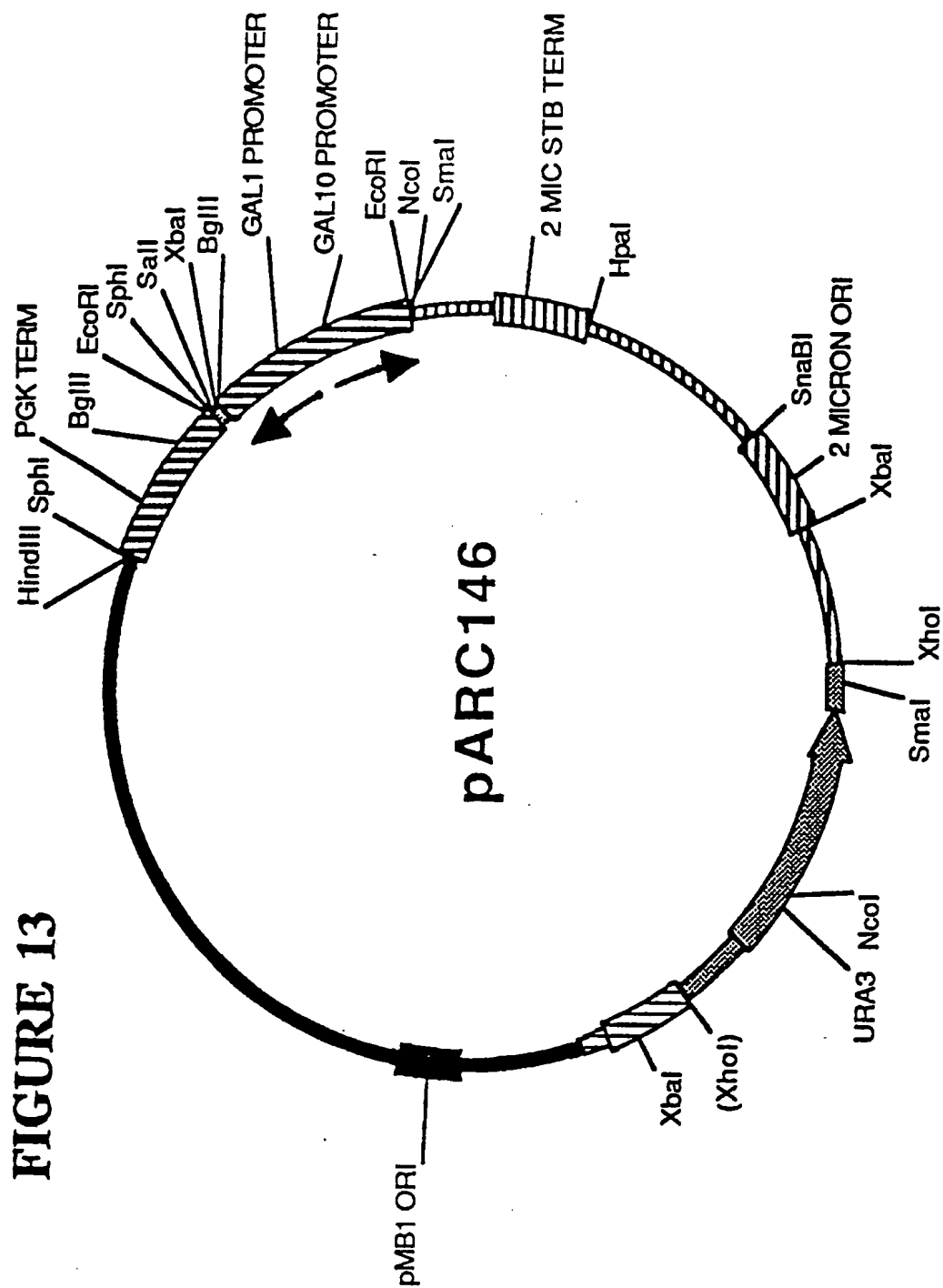
Nco I Bal I
CCACGCAGACCATGGCCA

FIGURE 11-4

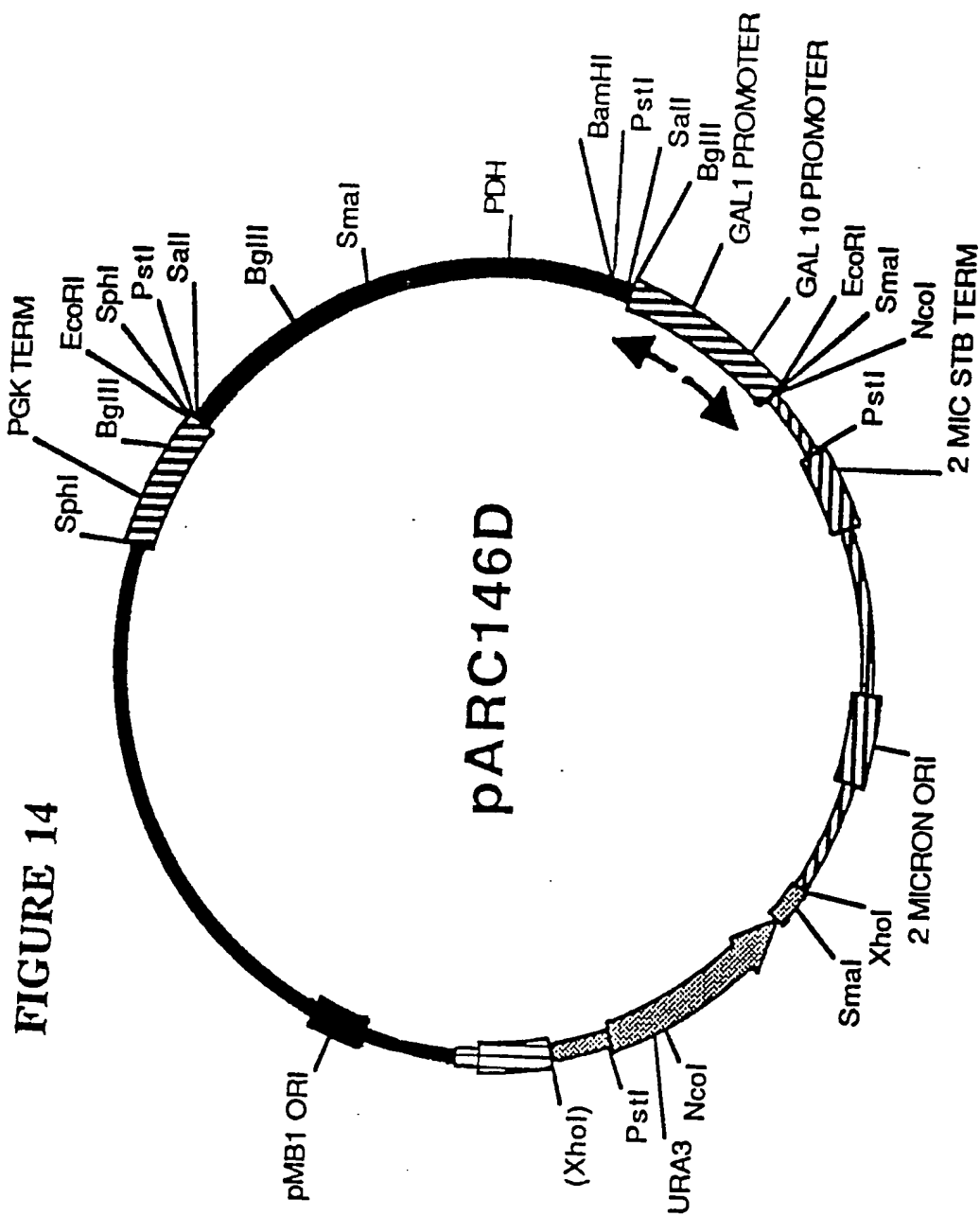
FIGURE 12



22



23



Sal I GAGG
TCGACGATGGAAAAAACCGTTGTGATTGGCGCAGGCTTGGTGGCCTGGCGCTGGCGATT 64
MetGluLysThrValValIleGlyAlaGlyPheGlyGlyLeuAlaLeuAlaIle

Pst I Bam HI
CGCCTGCAGCGGCAGGATCCCAACCGTACTGCTGGAGCAGCGGACAAGCCCGCGGT 124
ArgLeuGlnAlaAlaGlyIleProThrValLeuLeuGluGlnArgAspLysProGlyGly

CGGGCCTACGTCTGGCATGACCAGGGCTTTACCTTTGACCGCGGCGGACGGTGATCAC 184
ArgAlaTyrValTrpHisAspGlnGlyPheThrPheAspAlaGlyProThrValIleThr

GATCCTACCGCGCTTGAGCGGCTGTTCAACCTGGCCGCGCATGGAGGATTACGTC 244
AspProThrAlaLeuGluAlaLeuPheThrLeuAlaGlyArgArgMetGluAspTyrVal

AGGCTGCTGCCGGTAAACCCCTTCTACCGACTCTGCTGGGAGTCCGGGAAGACCCCTCGAC 304
ArgLeuLeuProValLysProPheTyrArgLeuCysTrpGluSerGlyLysThrLeuAsp

TATGCTAACGACAGCTTCGAGCTTGAGGCGCAGATTACCCAGTTCAACCCCGGACGTC 364
TyrAlaAsnAspSerPheGluLeuGluAlaGlnIleThrGlnPheAsnProArgAspVal

FIGURE 15-1

424 GAGGGCTACCGCGCTTCTGGCTTACTCCAGGCGGTATTCAGGAGGGATATTTCGCGC
GluGlyTyrArgArgPheLeuAlaTyrSerGlnAlaValPheGlnGluGlyTyrLeuArg

484 CTGGCAGCGTGCCGTTCCCTCTCTTTTCGCGACATGCTGCGCGCCGGCCGAGCTGCTT
LeuGlySerValProPheLeuSerPheArgAspMetLeuArgAlaGlyProGlnLeuLeu

Nru I

544 AAGCTCCAGGCGTGCGAGCGCTCTACCAAGTCGGTTTCGCGCTTTATTGAGGATGAGCAT
LysLeuGlnAlaTrpGlnSerValTyrGlnSerValSerArgPheIleGluAspGluHis

604 CTGCGGAGGCGCTTCTCGTTCCACTCCCTGCTGGTAGCGGCAACCCCTTCACCACTCG
LeuArgGlnAlaPheSerPheHisSerLeuLeuValGlyGlyAsnProPheThrThrSer

664 TCCATCTACACCCCTGATCCACGCCCTTGAGCGGAGTGGGGGTCTGGTTCCCTGAGGGC
SerIleTyrThrLeuIleHisAlaLeuGluArgGluTrpGlyValTrpPheProGluGly

724 GGCACCGGCGCTGGTGAACGGCATGGTGAAGCTGTTACCGATCTGGGCGGGAGATC
GlyThrGlyAlaLeuValAsnGlyMetValLysLeuPheThrAspLeuGlyGlyGluIle

Sma I

784 GAACTCAACGCCCGGTCTGAAGAGCTGGTGGCCGATAACCGCGTAAGCCAGGTCCGG
GluLeuAsnAlaArgValGluGluLeuValValAlaAspAsnArgValSerGlnValArg

FIGURE 15-2

CTCGCGGATGGTCGGATCTTTGACACCGACGCCGTAGCCTCGAACGCTGACGTGGTGAAC LeuAlaAspGlyArgIlePheAspThrAspAlaValAlaSerAsnAlaAspValValAsn	844
ACCTATAAAAGCTGCTCGGCACCATACCGGTGGGGCAGAACGGCGCACGGCTGGAG ThrTyrLysLysLeuLeuGlyThrIleProValGlyGlnLysArgAlaAlaArgLeuGlu	904
CGCAAGAGCATGAGCAAACTCGCTGTTTGTGCTCTACTTCGGCCCTGAACCAAGCCTCATTC ArgLysSerMetSerAsnSerLeuPheValLeuTyrPheGlyLeuAsnGlnProHisSer	964
CAGCTGGCGCACCATACCATCTGTTTGTGTTTGGTCCCCGCTACCGGGAGCTGATCGACGAGATC GlnLeuAlaHisHisThrIleCysPheGlyProArgTyrArgGluLeuIleAspGluIle	1024
Bgl II	
TTTACCGGCAGCGCGCTGGCGGATGACTTCTCGCTCTACCTGCACCTCGCCCTGCGTGACC PheThrArgGlnThrSerArgHisAlaTrpIleAlaIleLeuGlySerLeuPheIleGlu	1084
GATCCCTCGCTCGCGCCTCCCCCGTGGCGCCAGCTTCTACGTGCTGGCCCCGGTGCCGCAT AspProSerLeuAlaProProProCysAlaSerPheTyrValLeuAlaProValProHis	1144

FIGURE 15-3

1204 CTTGGCAACGGCCGCTGGACTGGCGCAGAGGGCCGAAGCTGCGCGACCGCATCTTT
LeuGlyAsnAlaProLeuAspTrpAlaGlnGlyProLysLeuArgAspArgIlePhe

1264 GACTACCTTGAAGAGCGCTATATGCCCGCCTGCGTAGCCAGCTGGTGACCCAGCGGATC
AspTyrLeuGluGluArgTyrMetProGlyLeuArgSerGlnLeuValThrGlnArgIle

1324 TTTACCCGGCAGACTTCACGACACGCTTGGATCGCGATCTTGGGATCGCTTTTCATCGAG
PheThrArgGlnThrSerArgHisAlaTrpIleAlaIleLeuGlySerLeuPheIleGlu

1384 CCGCCTTCGTTGACCCAAAGGCTTGTTCGCCGCAACGCGACACGACATTCAAACCTCTAC
ProProSerLeuThrGlnGlyLeuPheAlaAlaAsnAlaThrArgHisSerAsnLeuTyr

1444 CTGGTGGCCGAGGTACTCACCCTGGCGCGGCATTCTCGCGTAGTGGGCCCTCGCCGAA
LeuValAlaAlaGlyThrHisProGlyAlaGlyIleProGlyValValGlyLeuAlaGlu

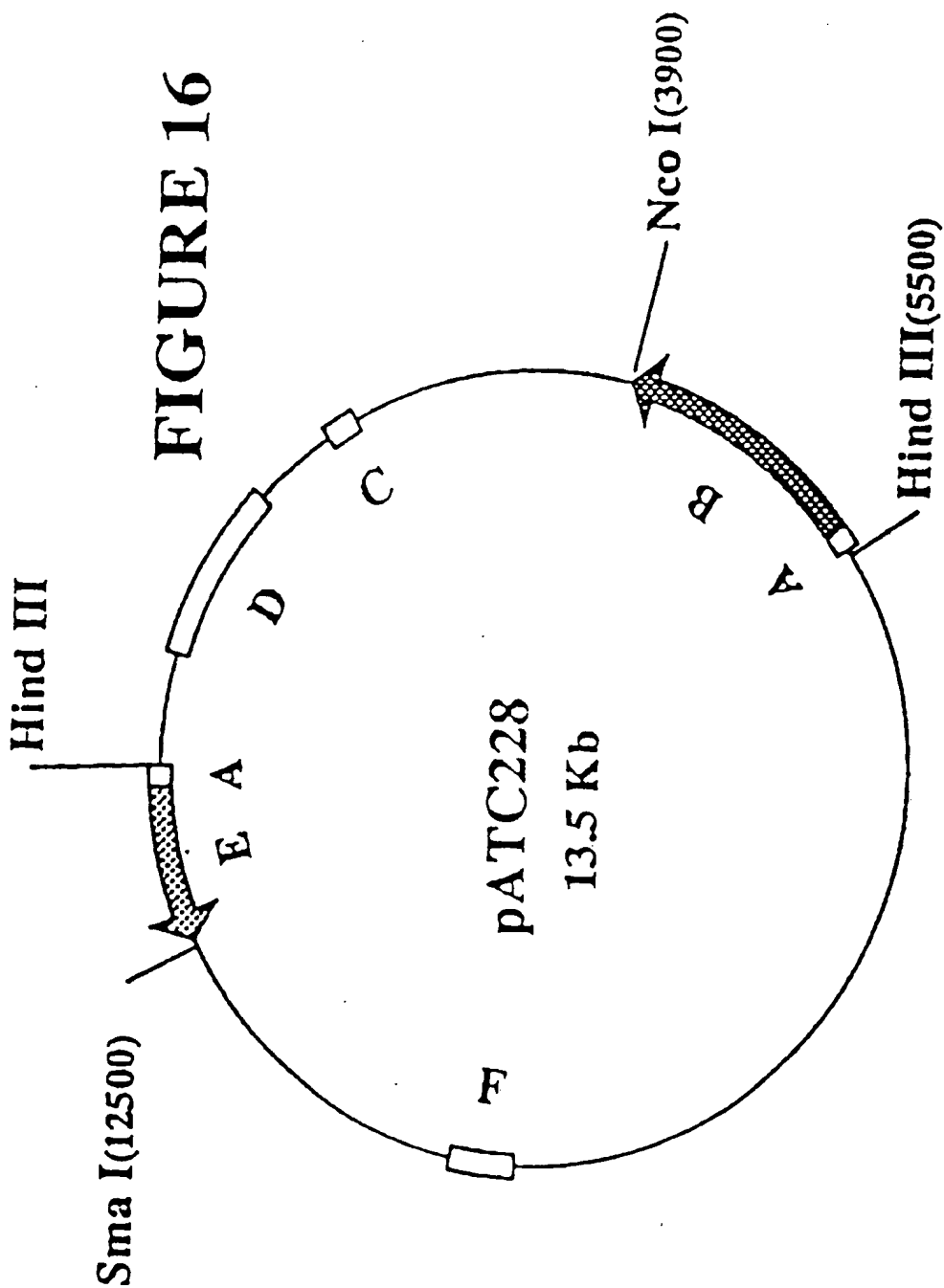
1504 AGCACCGCCAGCCTGATGATTGAGGATCTGCAATGAGCCAACCGCCGCTGCTTGACCAACG
SerThrAlaSerLeuMetIleGluAspLeuGln

Sal I
CCACGTCGACCATGGCCA

FIGURE 15-4

28

FIGURE 16



29

FIGURE 17

ATG GCT TCC TCA GTT CTT TCC TCT GCA GCA GTT GCC ACC CGC AGC	27
MET Ala Ser Ser Val Leu Ser Ser Ala Ala Val Ala Thr Arg Ser	
AAT GTT GCT CAA GCT AAC ATG GTG GCG CCT TTC ACT GGC CTT AAG	81
Asn Val Ala Ala Gln Ala Asn MET Val Ala Pro Phe Thr Gly Leu Lys	
TCA GCT GCC TCA TTC CCT GTT TCA AGG AAG CAA AAC CTT GAC ATC	135
Ser Ala Ala Ser Phe Pro Val Ser Arg Lys Gln Asn Leu Asp Ile	
ACT TCC ATT GCC AGC AAC GGC GGA AGA GTG CAA TGC ATG CAG	162
Thr Ser Ile Ala Ser Asn Gly Gly Arg Val Gln Cys MET Gln	

54

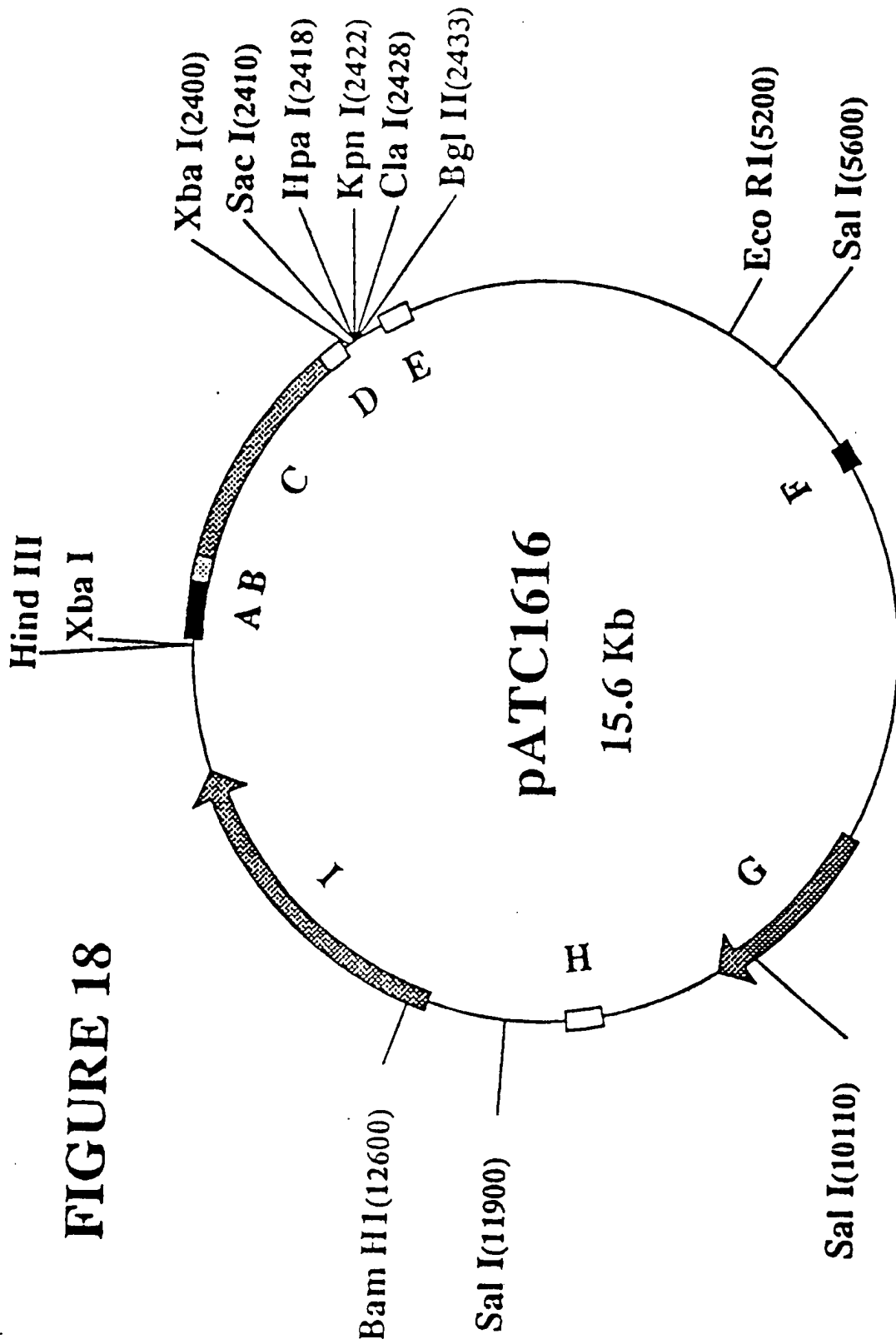
*

*

108

162

30



Sph I

AGGGAGTGAGAGCGTATCGTGAGGGATCTGATTTTAGTCGGCCGGCCTGGCCAACGGG 60
GCGCGCATGCGG
 MetArgAspLeuIleLeuValGlyArgGlyLeuAlaAsnGly

CTGATCGCCTGGCGTCTGCCCGCAGCGCTACCCGAGCTTAACCTGCTGCTGATCGAGGCC 120
 LeuIleAlaTrpArgLeuArgGlnArgTyrProGlnLeuAsnLeuLeuIleGluAla

GGGAGCAGCCCCGGGGAACCATACCTGGTCATTCCATGAAGACGATCTGACTCCCGGG 180
 GlyGlnProGlyGlyAsnHisThrTrpSerPheHisGluAspLeuThrProGly

CAGCAGCCTGGCTGGCCCCGCTGGTGGCCCCACGCCCTGGCCGGCTATGAGGTGCAGTTT 240
 GlnHisAlaTrpLeuAlaProLeuValAlaHisAlaTrpProGlyTyrGluValGlnPhe

CCCGATCTTCGCCGCTCGCCGCGGCTACTACTCCATTACCTCAGAGCGCTTGCC 300
 ProAspLeuArgArgLeuAlaArgGlyTyrTyrSerIleThrSerGluArgPheAla

GAGGCCCTGCATCAGGCGCTGGGGAGAACATCTGGCTAAACTGTTCGGTGAGCGAGGTG 360
 GluAlaLeuHisGlnAlaLeuGlyGluAsnIleTrpLeuAsnCysSerValSerGluVal

TTACCCAATAGCGTGGCCTTGCCAAACGGTGAGCGCTGCTGCCGGAGCGGTGATTGAC 420
 LeuProAsnSerValArgLeuAlaAsnGlyGluAlaLeuLeuAlaGlyAlaValIleAsp

FIGURE 19-1

32

480
GGACGGCGGTGACCGCCAGTTCGGCGATGCAACCGGCTATCAGCTCTTCTTGGTCAG
GlyArgGlyValThrAlaSerSerAlaMetGlnThrGlyTyrGlnLeuPheLeuGlyGln

540
CAGTGGCGGTGACACAGCCCCACGGCCTGACCGTACCGATCCTGATGGATGCCACGGTG
GlnTrpArgLeuThrGlnProHisGlyLeuThrValProIleLeuMetAspAlaThrVal

600
GCGCAGCAGCAGGGCTATCGCTTTGTCTACACGCTGCCGCTCTCCGCCGACACGCTGCTG
AlaGlnGlnGlyTyrArgPheValTyrThrLeuProLeuSerAlaaspThrLeuLeu

660
ATCGAGGATACGGCGCTACGCCAATGTCCCGCAGCGTGATGATAATGCCCTACGCCAGACG
IleGluaspThrArgTyrAlaasnValProGlnArgAspAsnAlaLeuArgGlnThr

720
GTTACCGACTATGCTCACAGCAAAGGTGGCAGCTGGCCAGCTTGAACGCCGAGGAGACC
ValThrAspTyrAlaHisserLysGlyTrpGlnLeuAlaGlnLeuGluArgGluGluThr

780
GGCTGTCTGCCGATTACCTGGCGGTGACATCCAGGCTCTGTGGGCCGATGCCCGCGGT
GlyCysLeuProIleThrTrpArgValThrSerArgLeuCysGlyProMetArgArgArg

840
GCCGCGTCGGGAATCGGGGCTGGGCTATTTCACCCCTACCCTGGCTATTCGCTGCCGCTG
AlaAlaSerGlyMetArgAlaGlyLeuPheHisProThrThrGlyTyrSerLeuProLeu

FIGURE 19-2

CGGTGGCCCTTGCCGACGCGATTGCCGACAGCCCGCGGCTGGCAGCGTTCCGCTCTAT 900
AlaValAlaLeuAlaAspAlaIleAlaAspSerProArgLeuGlySerValProLeuTyr

CAGCTCACCCGGCAGTTTGCCGAACGCCACTGGCGCAGGCAGGATTCTTCCGCCCTGCTG 960
GlnLeuThrArgGlnPheAlaGluArgHisTrpArgArgGlnGlyPhePheArgLeuLeu

AACCGGATGCTTTTCTGGCCGGCGGAGAGAACCGCTGGCGGTGATGCAGCGCTTT 1020
AsnArgMetLeuPheLeuAlaGlyArgGluGluAsnArgTrpArgValMetGlnArgPhe

TATGGGCTGCCGAGCCACCGTAGAGCGCTTTTACGCCGGTCCGGCTCTCTCTTTGAT 1080
TyrGlyLeuProGluProThrValGluArgPheTyrAlaGlyArgLeuSerLeuPheAsp

AAGCCCCGCATTTTGACGGGCAAGCCACCGGTTCCGCTGGCGAAGTCTGGCGGGCGCGC 1140
LysAlaArgIleLeuThrGlyLysProProValProLeuAlaLysSerGlyGlyArgArg

Bam HI
TGAACCATTTTCCCTGACAGACGAGATAAAGGATGAAAAAACCGTTGTGATTGGCGCAGG 1200
GATCCGATG

Pst I
CTTTGGTGGCCTGGCGCTGGCGGATTCCGCCTGCAG

FIGURE 19-3

34

TGAGTCGAGGAGCGCGCACCGCTATGCTAGTAAATAGTTTAATCGTCATCTTGACCGTT 60

ATTGCGATGGAGGCGCATCGCCCGGTTTACCCACCGCTACATTATGCACGGCTGGGGATGG 120

CGCTGGCATGAGCCACACCATACCCCGCGCAAGGCGGTATTGAGCTAAACGATCTCTTT 180

GCGGTGGTGTTTCCCGGCTGGCTATCCCGGCTGATTGCCGTGGGCA CGGGCGGTTTGG 240

CCCCTGCA GTGGCTGCGGCATGACGGTCTATGGCCTGCTCTACTTCCTGGTGCAC 300

Bam HI

GACGGTCTGGTGCA TCAGCGCTGGCCCTTCCACTGGATCCCCGCCGGGGCTACCTGAAG 360

CGGCTCTACGTGCGCCACCGCCTGCACCA CGCGGTGCGCGCGCGGAGGGCTGCGTCTCC 420

FIGURE 20-1

TTTCGGTTTATTACGCCCGCAAGCCTGCCGACCTACAGGCGATCCTGCGTGAACGTCAT 480
GGCCGCCCGCCTAAACGGGACGCTGCCAAAGACCGGCCGACGCGCGTCACCCCTCGTCG 540
TCTTCGCCCGAATAACCTGCCCGGTGCCGCCATCAGCATGGCAATTTTTTCACCTTTGC 600
TGGTGTGCTGGCGCGGATCCCAGGCGCTGCCTCCCGCGCTTTTACCTTAATACCGATCT 660
CCCGGTAGACGCTGCCGGCGGTGGCGATCGCCCAACGCGGAGCGCCGCCAGATCGTGTA 720
GCCCCGCCCTGGGAGGAGATGTAGTACGGCTCTGCGGCATCAATAAGCCTCCGCCACCGCG 780
CCAGCGCGGGCGATTCTCCCGCGCGGCATAGTTCTCCGGGGCCAGCCCGGCATCCTGCA 840

FIGURE 20-2

36

Sma I
GCCACTCGGGCAGATAGCAGCGGTCAATAGCCGCATCGTCAATAATATCCCGGCCA 900

TATTTCGTCAGCTGGAAGGCCAGCCCCAGATCGCAGGCGGATCCAGC

FIGURE 20-3

Nco I

60 TGAGTCGACGAGCGCGCACCGCCATGGTACTAAATAGTTAAATCGTCATCTTGACCGTT
MetValLeuAsnSerLeuIleValIleLeuThrVal

120 ATTGCGATGGAGGCGATCGCCGCGTTTACCCACCGCTACATTAATGCACGGCTGGGGATGG
IleAlaMetGluGlyIleAlaAlaPheThrHisArgTyrIleMetHisGlyTrpGlyTrp

180 CGCTGGCATGAGCCACACCATACCCCGCGCAAGGCGTATTTGAGCTAAACGATCTCTTT
ArgTrpHisGluProHisThrProArgLysGlyValPheGluLeuAsnAspLeuPhe

240 GCGGTGGTGTTCGCCGGGTGCTATCGCGCTGATTGCCGTGGCACCAGCGCGGCTTGG
AlaValValPheAlaGlyValAlaIleAlaLeuIleAlaValGlyThrAlaGlyValTrp

300 CCCCTGCAGTGGATTGGCTGCGGCATGACGGTCTATGGCCTGCTCTACTTCCTGGTGCAC
ProLeuGlnTrpIleGlyCysGlyMetThrValTyrGlyLeuLeuTyrPheLeuValHis

Bam HI

360 GACGGTCTGGTGCATCAGCGCTGGCCCTTCCACTGGATCCCCGCCGGGGTACCTGAAG
AspGlyLeuValHisGlnArgTrpProPheHisTrpIleProArgArgGlyTyrLeuLys

420 CGGCTCTACGTCGCCCCACCGCCTGCACCACCGCGGTGCGCGCGCGGAGGGCTGCGTCTCC
ArgLeuTyrValAlaHisArgLeuHisHisAlaValArgGlyArgGluGlyCysValSer

FIGURE 21-1

38

TTCCGGTTTATTACGCCCGCAAGCCTGCCGACCTACAGGCGATCCTGCGTGAACGTTCAT 480
PheGlyPheIleTyrAlaAArgLysProAlaAspLeuGlnAlaIleLeuArgGluArgHis
GGCCGCCCGCCTAAACGGGACGCTGCCAAAGACGGCCGGACGCGGCGTCAACCCTCGTCG 540
GlyArgProProLysArgAspAlaAlaLysAspArgProAspAlaAlaSerProSerSer
TCTTCGCCCCGAATAACCTGCCCCCGGTGCCGCCCATCAGCATGGCAATTTTTTCACCTTTGC 600
SerSerProGlu
TGGTGCTGGCGGCGATCCAGGCGCTGCCCTCCCGCCGCTTTTACCTTAATACCGATCT 660
CCCCGTAGACGCTGCGGGCGGTGGCGATCGCCACGCGGAGCGCCGCGAGATCGTGTA 720
GCCCGGCCCTGGGAGGAGATGTAGTACGGCTCTGCGGCGATCAATAAGCCTCCGCCACCGCG 780
CCAGCGCGGGCGGATTCTCCCGCGCGGCATAGTTCTCCGGGGCCAGCCCGGCATCCTGCA 840

FIGURE 21-2

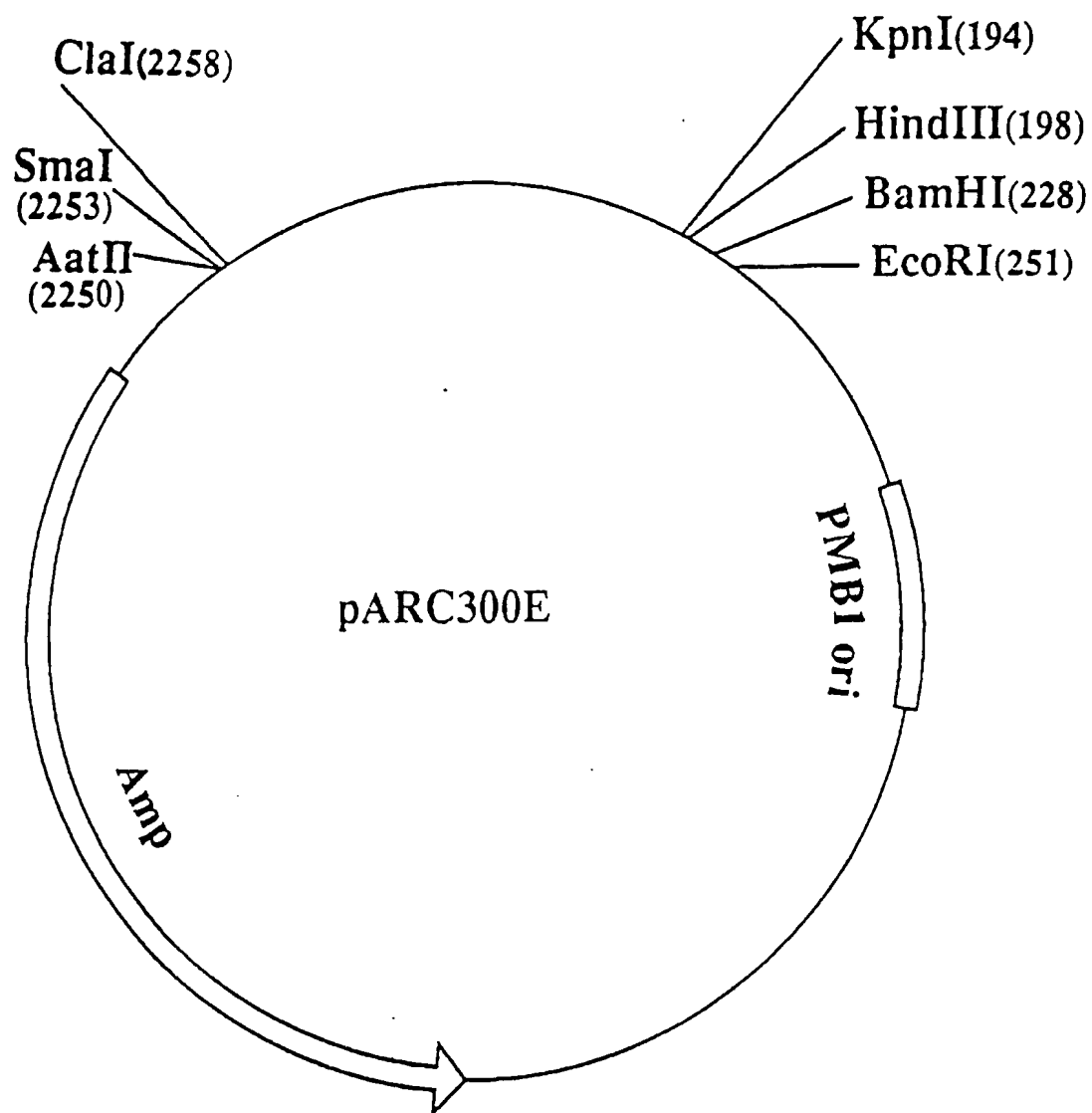
Sma I

GCCACTCGGCGGCAGATAGCAGCGGTCAATAGCCGCATCGTCAATAATATCCCGGGCCA 900

TATTTCGTCAGCTGGAAGGCCAGCCCCAGATCGCAGGCGGATCCAGC

FIGURE 21-3

FIGURE 22



41

FIGURE 23

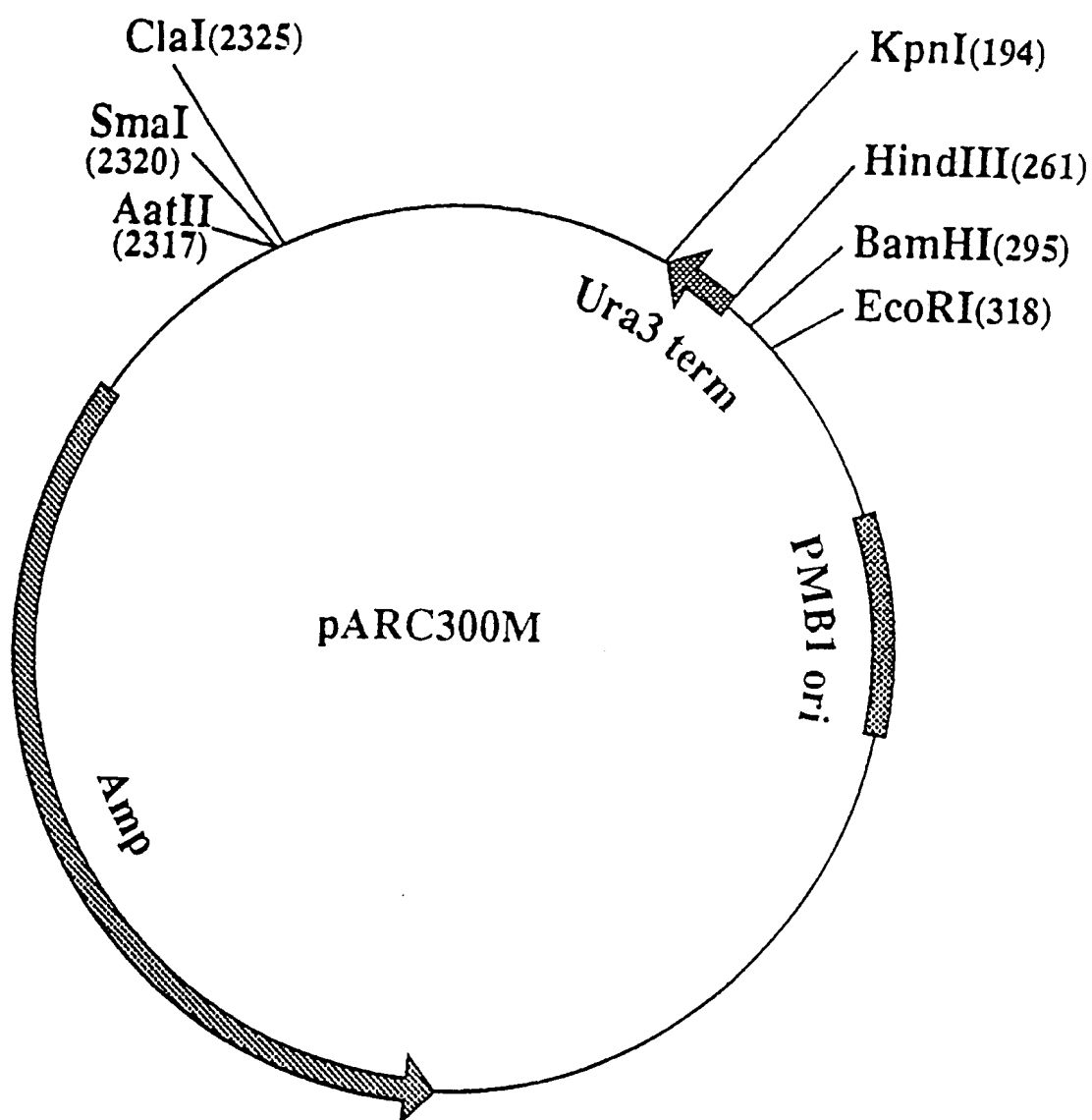
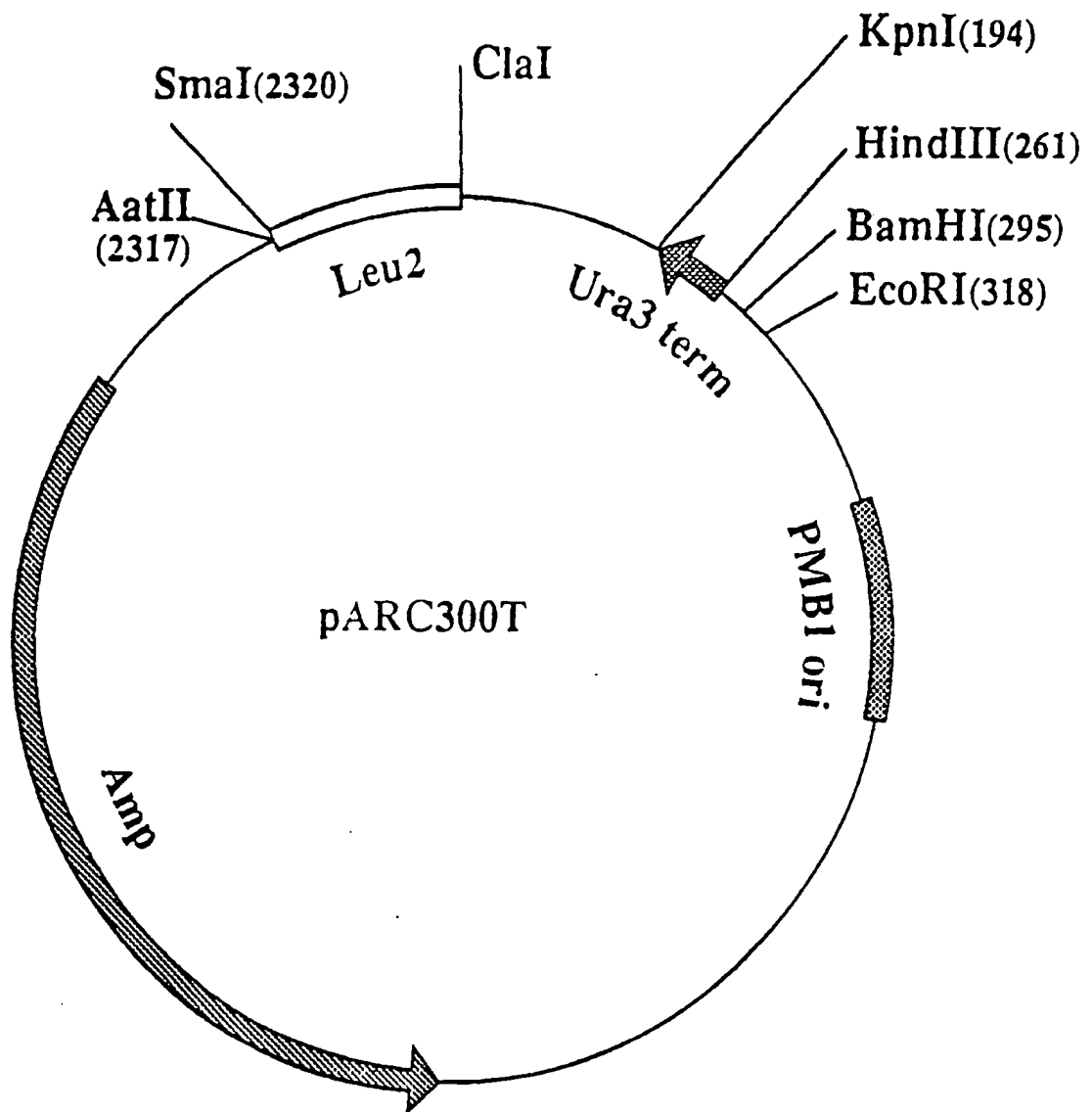


FIGURE 24



43

ATGAGCCATTGTGGCACCGCCGCTCTACAGTCATGCGGTGCGTGCATGCC 60
METSerHisPheAlaIleValAlaProProLeuTyrSerHisAlaValAlaLeuHisAla

CTGGCGCTGCAGATGGCCCAACGCGGCCACCGGGTGACCTTTCTCACC GGCAACGTCGCC 120
LeuAlaLeuGluMETAlaGlnArgGlyHisArgValThrPheLeuThrGlyAsnValAla

TCGCTGGCAGACGAGAAACGAGCGGGTGCGGTCTCTATCCACTTCCCGCCAGCGTGCAA 180
SerLeuAlaGluGlnGluThrGluArgValAlaPheTyrProLeuProAlaSerValGln

CAGGCCCAGCGCAACGTCCAGCAGCAGAGTAACGGCAACCTGTCTGCGGCTGATTGCGGCC 240
GlnAlaGlnArgAsnValGlnGlnSerAsnGlyAsnLeuLeuArgLeuIleAlaAla

ATGTCATCCCTGACCGATGTGCTCTGCGAGCAGTTGCCCGCTATTCTACAGCGGCTGGCG 300
METSerSerLeuThrAspValLeuCysGlnGlnLeuProAlaIleLeuGlnArgLeuAla

GTGGACGCGCTGATTGTCTGATGAGATGGAGCCCCCGGAAGCCTGGTCGCCGAGCGCTG 360
ValAspAlaLeuIleValAspGluMETGluProAlaGlySerLeuValAlaGluAlaLeu

GGACTACCATTTATCTCTATTGCCCTGCGCGCTGCCGGTCAACCGGAGCTGCCGCTGCCG 440
GlyLeuProPheIleSerIleAlaCysAlaLeuProValAsnArgGluLeuProLeuPro

FIGURE 25-1

GTGATGCCGTTTCACTACGCCGAGGATAAGAGAGCCCCGTGCGGTTTTCAGGTCAGCGAA 500
ValMETProPheHisTyrAlaGluAspLysArgAlaArgPheGlnValSerGlu

CGGATCTACGATGCGCTGATGTACCCGGACGGGCAGACGATCCTGCGCCACGCCGACGCGC 560
ArgIleTyrAspAlaLeuMETTyrProHisGlyGlnThrIleLeuArgHisAlaGlnArg

TTTGTTGCCGAGCCGAGCGGTCTCGACGAGTGTCTCTCGCCCCCTGGCGCAGATTAGC 620
PheGlyLeuProGluArgArgArgLeuAspGluCysLeuSerProLeuAlaGlnIleSer

CAGTCCGTTCCGGCCCTCGACTTCCACGCCGGCGCTGCCGAACGTGTTTACCTACGTG 680
GlnSerValProAlaLeuAspPheProArgArgAlaLeuProAsnCysPheThrTyrVal

GGAGCACTGCGCTATCAGCCCCCGCCGAGGTAGAACGCTCGCCACGCAGCACGCCGCGG 740
GlyAlaLeuArgTyrGlnProProGlnValGluArgSerProArgSerThrProArg

ATCTTTGCCCTCGCTGGGCACCCCTCCAGGGCCACCGTCTACGCCCTGTTTCAGAAGATCGCC 800
IlePheAlaSerLeuGlyThrLeuGlnGlyHisArgLeuArgLeuPheGlnLysIleAla

CGCGCCTGTGCCAGCGTGGCGCGGAGGTGACCATTTGCCCACTGCGATGGCCTGACGCCC 860
ArgAlaCysAlaSerValGlyAlaGluValThrIleAlaHisCysAspGlyLeuThrPro

FIGURE 25-2

GCCAGGCCGACTCGCTCTACTGCGGCGCGACGGAGGTGGTCAGCTTTGTTCGACCAGCCG 940
AlaGlnAlaAspSerLeuTyrCysGlyAlaThrGluValSerPheValAspGlnPro

CGCTACGTTGCCGAGGCTAATCTGGTGATCACCCACGGCGGTCTCAATACCGTACTGGAT 1000
ArgTyrValAlaGluAlaAsnLeuValIleThrHisGlyGlyLeuAsnThrValLeuAsp

GCGCTGGCTGCCGCGACGCCGGTGCTGGCGGTGCCACTCTCTTCGACCAGCCCGCCGTG 1060
AlaLeuAlaAlaAlaThrProValLeuAlaValProLeuSerPheAspGlnProAlaVal

GCTGCCCGGCTGGTCTATAACGGGCTGGGTGCGCGGTATCGCGCTTTGCCAGACAGGAG 1140
AlaAlaArgLeuValTyrAsnGlyLeuGlyArgArgValSerArgPheAlaArgGlnGln

ACGCTGGCGGATGAGATTGCCCAACTGCTGGGGGATGAGACGCTGCATGAGCGTGTGGCG 1200
ThrLeuAlaAspGluIleAlaGlnLeuLeuGlyAspGluThrLeuHisGlnArgValAla

ACGGCCCGCAGCAGCTTAACGACGCCGGGGCAGCCCCCGTTGCGGGCAGCCCTGATTGA 1260
ThrAlaArgGlnGlnLeuAsnAspAlaGlyGlyThrProArgCysGlyAspProAsp

FIGURE 25-3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01458

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07H 15/12; C12P 21/00, 23/00, 57/00; C12N 15/52, 15/70, 15/74, 15/81 US CL: 536/27; 435/67, 69.1, 166, 172.3, 320		
II. FIELDS SEARCHED		
Minimum Documentation Searched †		
Classification System	Classification Symbols	
U.S.	435/67, 69.1, 166, 172.3, 320; 536/27; 935/35, 60, 64, 67, 69, 72, 73	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
U.S. PTO Automated Patent System; DIALOG files: BIOTECH, PATENTS; Gen BANK/UEMBL and PIR/SWISS PROT See Attachment for search terms		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ‡		
Category *	Citation of Document, † with indication, where appropriate, of the relevant passages ‡	Relevant to Claim No. ‡
Y	Plants Today; Volume 1, Number 1, Issues January-February 1988, Bryant, "Putting genes into plants", pages 23-28. See page 26.	5, 12, 16, 23, 25, 28, 38, 42, 52, 56, 65, 69 and 74-76
Y	Weed Science, Volume 26, Issued 1978, Bartels et al, "Inhibition of Carotenoid Synthesis by fluridone and norflurazon", pages 198-203. See entire document.	74-76
Y	Plant Physiology (Bethesda, USA), Volume 88, Number 2, Issued 1988, Sagar et al, "Light effects on several chloroplast components in norflurazon-treated pea seedlings", pages 340-347. See entire document.	74-76
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: †</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
29 May 1991		07 AUG 1991
International Searching Authority		Signature of Authorized Officer
TSA/IUS		P. Rhodes

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

norflurazon resistance; carotenoid or geranylgeranyl or
phytoene or lycopene or carotene or zeaxanthin; erwinia;
Rhodobacter; sequence or clone.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
<u>X</u> Y	Journal of Biological Chemistry; Volume 264, Number 22; Issued 05 August 1989; Bartley et al; "Carotenoid biosynthesis in photosynthetic bacteria genetic characterization of the Rhodobacter capsulatus CrtI protein"; pages 13109-13113. (see entire document)	19-22, 24 33, 74-76
Y,P	Journal of Biological Chemistry; Volume 265, Number 14; Issued 15 May 1990; Armstrong et al; "Genetic and biochemical characterization of carotenoid biosynthesis mutants of Rhodobacter capsulatus"; pages 8329-8338. (see entire document)	1-73
Y,P	Journal of Biological Chemistry; Volume 265, Number 26; Issued 15 September 1990; Bartley et al; "Carotenoid desaturases from Rhodobacter capsulatus and Neurospora crassa are structurally and functionally conserved and contain domains homologous to flavoprotein disulfide oxidoreductases"; pages 16020-16024. (see entire document)	1-73
Y	European Journal of Biochemistry; Volume 184, Number 2; Issued September 1989; Schmidt et al; "Immunological detection of phytoene desaturase in algae and higher plants using an antiserum raised against a bacterial fusion-gene construct"; pages 375-378. (see entire document)	1-76
Y	Journal of General Microbiology; Volume 130; Issued 1984; Thiry; "Plasmids of the epiphytic bacterium Erwinia uredovora"; pages 1623-1631. (see entire document)	1-76
<u>X</u> , P Y	Gene; Volume 91, Number 1; Issued 02 July 1990; Schmidt et al; "Cloning and nucleotide sequence of the crtI gene encoding phytoene dehydrogenase from the cyanobacterium Aphanocepsa PCC6714"; pages 113-117. (see entire document)	19-22, 24 18, 25-33 44-46, 57-59 70-72, 74-76
Y,P	Proceedings of the National Academy of Sciences USA; Volume 87; Issued December 1990; Armstrong et al; "Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes"; pages 9975-9979. (see entire document)	1-73

III. DOCUMENTS CONSIDERED 3E RELEVANT (CONTINUED FROM THE SECOND S ET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	FEMS Microbiology Letters; Volume 78; Issued 01 March 1991; Schnurr et al; "Mapping of a carotenogenic gene cluster from <i>Erwinia herbicola</i> and functional identification of six genes"; pages 157-162. (see entire document)	1-4,8-11,19-22, 34-37,48-51, and 61-64
Y	Journal of Cellular Biochemistry; Volume 12C; Issued 1988; Bennetzen et al; "Structure and protective role of the carotenoid synthesis gene(s) of <i>Erwinia stewartii</i> "; page 246. (see abstract Y101)	1-76
Y	Trends in Genetics; Volume 4, Number 8; Issued August 1988; Botterman et al; "Engineering herbicide resistance in plants"; pages 219-222. (see entire document)	74-76
Y	Bio/Technology; Volume 6; Issued August 1988; Hinchee et al; "Production of transgenic soybean plants using <i>Agrobacterium</i> -mediated DNA transfer"; pages 915-922. (see entire document)	74-76
X Y	Molecular and Cellular Biology; Volume 9, Number 3; Issued March 1989; Nelson et al; "Molecular cloning of a <i>Neurospora crassa</i> carotenoid biosynthetic gene (Albino-3) regulated by blue light and the products of the white collar genes"; pages 1271-1276. (see entire document)	1,3-4,6 2,5,7,14-17, 29-32,44-46, 57-59,70-72

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	Current Microbiology, Volume 15, Issued 1987, Pemberton et al, "Expression of Rhodopseudomonas sphaeroides carotenoid photopigment genes in phylogenetically related nonphotosynthetic bacteria", pages 67-71. See entire document.	1, 3-4, 6, 8, 10 11, 13-15, 19 21-22, 24, 26- 27, 29, 34, 36- 37, 39-41, 43- 44, 48, 50-51, 53-55, 57, 61, 63-64, 66-68, 70 72, 5, 7, 9, 12, 16 18, 20, 23, 25, 28, 30-33, 35, 38, 42, 45-47, 49, 52, 56, 58- 60, 62, 65, 69 71-73
Y	Phytopathology, Volume 79, Number 2, Issued February 1989, Daub et al, "The role of carotenoids, in resistance of fungi to cercosporin", pages 180-185. See entire document.	74-76
Y	Journal of Phycology, Volume 23, Number 1, Issued 1987, Ben-Amotz et al, "Massive accumulation of phytoene induced by norflurazon in <u>Dunaliella-bardawil</u> (chlorophyceae) prevents recovery from photoinhibition", pages 176-181. see entire document.	74-76

III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND

EET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
<u>X</u> Y	Journal of Bacteriology; Volume 168, Number 2; Issued November 1986; Perry et al; "Cloning and Regulation of Erwinia herbicola Pigment Genes"; pages 607-612. (see entire document)	1-4, 6-11, 13-15, 17-22, 24, 26-27, 29-30, 33-37, 39-41, 43-45, 47-51, 53-55, 57-58, 60, 64, 66-68, 70-71, and 73 5, 12, 16, 23, 25, 28 31-32, 38, 42, 46, 52, 54-56, 59, 65, 69, 72, 74-76
Y	Journal of Bacteriology; Volume 170, Number 10; Issued October 1988; Tuveson et al; "Role of Cloned Carotenoid Genes Expressed in Escherichia coli in Protecting against Inactivation by Near-UV Light and Specific Phototoxic Molecules"; pages 4675-4680. (see entire document)	1-76
A	Journal of Bacteriology; Volume 171, Number 9; Issued September 1989; Tichy et al; "Genes Downstream from pucB and pucA are Essential for Formation of the B800-850 Complex of Rhodobacter capsulatus"; pages 4914-4922. (see entire document)	1-76
X, P	EP A 0,393,690 (MISAWA ET AL) 24 October 1990. (see entire document)	1-76
<u>X</u> , P Y	Journal of Bacteriology; Volume 172, Number 12; Issued December 1990; Misawa et al; "Elucidation of the Erwinia uredovora Carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli"; pages 6704-6712. (see entire document)	1-4, 6-11, 13-15, 17-22, 24, 26-27, 29-30, 33-37, 39-41, 43-45, 47-51, 53-55, 57-58, 60, 64, 66-68, 70-71, and 73 5, 12, 16, 23, 25, 28 31-32, 38, 42, 46, 52, 54-56, 59, 65, 69, 72, 74-76
Y	Molecular and General Genetics; Volume 213, Number 1; Issued July 1988; Giuliano et al; "A genetic-physical map of the Rhodobacter capsulatus carotenoid biosynthesis gene cluster"; pages 78-83. (see entire document)	1-76
<u>X</u> Y	Molecular and General Genetics; Volume 216, Number 2/3; Issued April 1989; Armstrong et al; "Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of Rhodobacter capsulatus"; pages 254-268. (see entire document)	1, 3-6, 8, 10-19, 21-34, 36-48, 50-61, and 63-73 2, 7, 9, 20, 35, 49, 62
A	Molecular and General Genetics; Volume 218, Number 1; Issued July 1989; Young et al; "Genetic evidence for superoperonal organization of genes for photosynthetic pigments and pigment-binding proteins in Rhodobacter capsulatus"; pages 1-12. (see entire document)	1-76

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

Zeitschrift fur Naturforschung, Volume 45c,
Issued March 1990, Chamovitz et al, "Cloning a gene
coding for norflurazon resistance in cyanobacteria"
pages 482-486. See entire document.

74-76

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:
3. ☐ Claim numbers because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

The claims present a plurality of mutually exclusive independent inventions as follows:

- I. Claims 1-7 and 17, drawn to a structural gene for geranylgeranyl pyrophosphate synthase, and vectors containing same and a first method of use to make geranylgeranyl pyrophosphate synthase protein, classified in Classes 435 and 536, subclasses 69.1 and 320 and subclass 27, respectively, for example.
- II. Claims 8-12 and 18, drawn to a structural gene for phytoene synthase and vectors containing same, classified in Class 435 and 536, subclasses 320 and 27, respectively, for example.
- III. Claim 13, drawn to a method of use for the product of Group II to make phytoene synthase protein, classified in Class 435, subclass 69.1, for example.
- IV. Claims 14-16, drawn to a method of making the compound phytoene, classified in Class 435, subclass 166, for example.
- V. Claims 19-23 and 33, drawn to a structural gene for phytoene dehydrogenase-4H and vectors containing same, classified in Class 435 and 536, subclasses 320 and 27, respectively, for example.
- VI. Claims 24-25, drawn to a first method of use for the product of Group V to make phytoene dehydrogenase-4H protein, classified in Class 435, subclass 69.1, for example.
- VII. Claims 26-28, drawn to a first method of making lycopene, classified in Class 435, subclass 166, for example.
- VIII. Claim 29-32, drawn to a second method of making lycopene, classified in Class 435, subclass 166, for example.
- IX. Claims 34-38 and 47, drawn to a structural gene for lycopene cyclase and vectors containing same, classified in Class 435 and 536, subclasses 320 and 27, respectively, for example.
- X. Claim 39, drawn to a method of use for the product of Group IX to make lycopene cyclase protein, classified in Class 435, subclass 69.1, for example.
- XI. Claim 40-43, drawn to a first method of making beta-carotene, classified in Class 435, subclass 67, for example.
- XII. Claim 44-46, drawn to a second method of making beta-carotene, classified in Class 435, subclass 67, for example.
- XIII. Claims 48-52 and 60, drawn to a structural gene for beta-carotene hydroxylase and vectors containing same, classified in Class 435 and 536, subclasses 320 and 27, respectively, for example.

Attachment to Form PCT/ISA/210, Part VI.VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING
(continuation)

- XIV. Claim 53, drawn to a method of use for the product of Group XIII to make beta-carotene hydroxylase protein, classified in Class 435, subclass 69.1, for example.
- XV. Claims 54-56, drawn to a first method of making zeaxanthin, classified in Class 435, subclass 67, for example.
- XVI. Claim 57-59, drawn to a second method of making zeaxanthin, classified in Class 435, subclass 67, for example.
- XVII. Claims 61-65 and 73, drawn to a structural gene for zeaxanthin glycosylase and vectors containing same, classified in Class 435 and 536, subclasses 320 and 27, respectively, for example.
- XVIII. Claim 66, drawn to a method of use for the product of Group XVII to make zeaxanthin glycosylase protein, classified in Class 435, subclass 69.1, for example.
- XIX. Claims 67-69, drawn to a first method of making zeaxanthin diglucoside, classified in Class 435, subclass 67, for example.
- XX. Claim 70-72, drawn to a second method of making zeaxanthin diglucoside, classified in Class 435, subclass 67, for example.
- XXI. Claims 74-76, drawn to a second method of use for the product of Group V to make plants resistant to the herbicide norflurazon, classified in Class 435, subclass 172.3, for example.